University of Alberta

Quantification of *ToxB* Gene Expression by Isolates of *Pyrenophora tritici-repentis* and Microscopic Analysis of the Interaction with their Wheat Host

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Plant Science

Department of Agricultural, Food, and Nutritional Science

Edmonton, Alberta
Spring 2007

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ISBN: 978-0-494-29927-2
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ISBN: 978-0-494-29927-2

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Abstract

Tan spot, caused by the fungus *Pyrenophora tritici-repentis*, is an important disease of wheat. The fungus produces Ptr ToxB, a protein toxin that causes chlorosis in sensitive wheat and is encoded by the *ToxB* gene. Using qRT-PCR, transcription of *ToxB* homologs found in virulent, low virulence and avirulent isolates of the fungus was compared in conidia, mycelia and after inoculation onto resistant and susceptible wheat genotypes. Expression of the gene was greatest in the virulent isolate, followed by the low virulence and avirulent isolates. Gene expression was correlated to the development of chlorosis in the susceptible wheat genotype, confirming the importance of Ptr ToxB in disease development. Microscopic analysis revealed that while all isolates could penetrate host epidermal cells, the virulent isolate produced significantly greater numbers of appressoria more quickly than the low virulence and avirulent isolates, suggesting that the basic pathogenic ability of these isolates is not the same.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. Stephen E. Strelkov for his suggestions and financial support, and to my committee members, Dr. Jocelyn A. Ozga, Dr. Kelly Turkington and Dr. Randolph S. Currah for their encouragement and suggestions.

I wish to thank Dr. Lakhdar Lamari for his gift of fungal isolates, Dr. Urmila Basu for her technical support in the qRT-PCR analysis, and Dr. Stephen Moore for allowing us to use his qRT-PCR machine.

I would also like to thank Mr. Rakesh Bhatnagar and Mr. Randy Mandryk for their technical assistance in the microscopy studies, and Dr. Rong-Cai Yang, Dr. Ellen Macdonald, and the staff at the Training Consulting Centre for their assistance with the statistical analysis

I also wish thank all of the members of Dr. Strelkov's laboratory, Dr. Ozga's laboratory, and Dr. Nat Kav's laboratory, whom I worked with throughout my graduate studies.

Finally, I would like to thank to my family in Japan and my partner, Mr. Isao Kurosawa, for their support during my studies.

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1.0 Introduction

1.1 Biology of the Pathogen

1.1.1 Outbreaks of the Disease Worldwide

The homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) causes tan spot of wheat, which has become an important disease worldwide (Hosford 1982; Lamari and Bernier 1989b; Strelkov and Lamari 2003). Also known as yellow leaf spot, eye spot, yellow leaf blotch, leaf blotch, and wheat leaf blight (Hosford 1982), the disease has been reported to occur in at least 21 countries (Hosford 1982; Tekauz et al. 1982). Major areas of disease occurrence include the wheat production areas of Australia, Nepal, South America (Brazil, Uruguay, and Argentina), North America (the Great Plains and Mexico), and the wheat centre of origin (the Fertile Crescent and the Caucasus region) (Lamari et al. 2003). Recently, the disease was also found in Pakistan and Syria (Ali and Francl 2001; Orabi et al. 2002).

Tan spot can cause substantial yield losses in severe epidemics and can have a great economic impact on wheat production. Yield losses associated with the disease can vary based on the growth stage of wheat (Rees et al. 1982). In greenhouse experiments, yield losses were highest when plants were inoculated at the boot and flowering stages of development (Shabeer and Bockus 1988). In the field, yield losses of 3-50 % have been reported in most wheat growing areas (Hosford 1982; Mehta and Gaudêncio 1991; Rees and Platz 1983), but losses as high as 75 % have also been

reported (Duff 1954). Rees et al. (1982) reported yield losses of about 49 % in Australia. In Paraná (Brazil), a 36 % yield loss was reported as a result of infection by *P. tritici-repentis* (Mehta and Gaudêncio 1991). The first large scale tan spot epidemic in western Canada occurred in spring wheat in 1974 (Tekauz 1976). In 1982, the first severe seedling infections were reported in southern Manitoba and southwestern Saskatchewan (Tekauz et al. 1982). Since then, tan spot of wheat has been considered one of the most important leaf spot diseases in western Canada. This increased incidence is believed to have resulted from changes in soil conservation practices by farmers, which retain infected wheat stubble and provide a source of inoculum for subsequent crops (Lamari and Bernier 1989a; 1989b; Strelkov and Lamari 2003; Tekauz 1976).

1.1.2 Disease Cycle and Development

Tan spot of wheat is a polycyclic disease with sexual and asexual stages (Fig. 1-1). *Pyrenophora tritici-repentis* overwinters as pseudothecia on wheat stubble in the field. In early spring, the sexual fruiting bodies mature and produce ascospores, which serve as the primary inoculum (Menzies and Gilbert 2003; Wiese 1987). Disease severity and yield losses have been correlated with the local level of primary inoculum (Pfender et al. 1993). The sexual stage of the disease begins with the development of the sexual organs, the antheridia (male) and ascogonia (female). The nuclei in the antheridia migrate into the ascogonia via the trichogyne. The antheridial and ascogonial nuclei pair up and undergo karyogamy. After that, the fusion nucleus

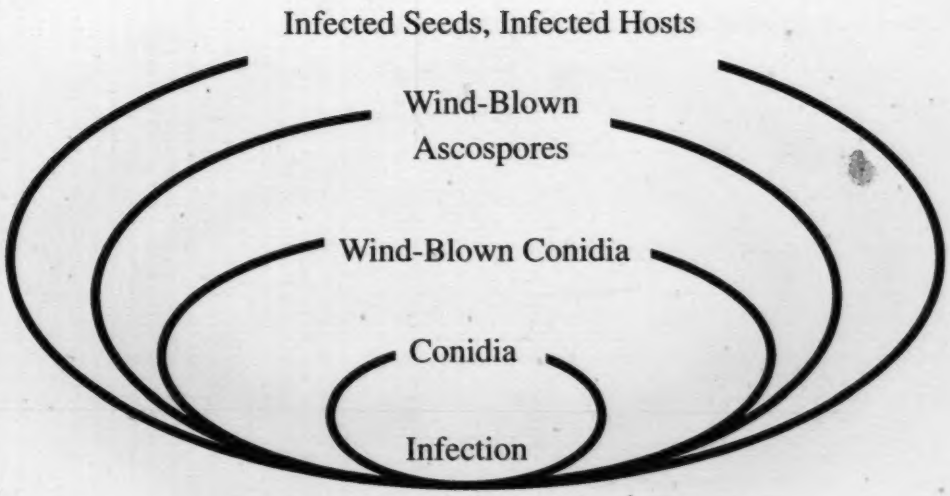

Figure 1-1. Disease cycle of tan spot of wheat (adapted from De Wolf et al. 1998)

enlarges and goes through meiosis, forming eight ascospores in two groups of four in an ascus (Wehmeyer 1954). The mature asci swell up and burst under high relative humidity. The ascospores are wind-dispersed over short distances in the spring and early summer, and produce the first lesions on wheat seedlings or other hosts. In general, conidia are produced on older lesions and serve as secondary inoculum during the growing season, and are also dispersed by wind (Menzies and Gilbert 2003; Schilder and Bergstrom 1992; Wiese 1987). Under favorable conditions, conidia can repeat the infection cycle several times during a single season. Infected crop residues and stubble remain as a source of primary inoculum to infect wheat in subsequent seasons. Infected wheat seeds are not an important source of inoculum (Menzies and Gilbert 2003; Wiese 1987).

1.1.3 Epidemiology

The incidence of tan spot of wheat has been associated with the duration of wet periods, wind-speed, photoperiod, and favorable temperatures (De Wolf et al. 1998; Hosford et al. 1987; Platt and Morrall 1980a). Additionally, it has been related to the level of cultivar susceptibility, the growth stage of wheat, and the virulence of *P. tritici-repentis*. Infected older leaves on the lower plant show more severe spotting than younger leaves on the upper plant (De Wolf et al. 1998; Hosford et al. 1987). Rees et al. (1988) reported that the reaction of wheat to the pathogen in greenhouse studies at the 2-leaf stage was similar to that observed under field conditions.

For germination of conidia and infection of the host, the pathogen requires periods of continuous wetness (Francl 1998; Hosford et al. 1987). According to Hosford et al. (1987), the pathogen requires wet periods of 6-12 hours on susceptible wheat cultivars, and more than 48 hours on resistant wheat cultivars. The ascospores of the pathogen are dispersed short distances, primarily by wind, under rainfall conditions or high relative humidity (RH). Schilder and Bergstrom (1992) found that most ascospores were collected within 0.35 m of the inoculum source, but some were collected as far as 11 m away. Pseudothecium formation by the pathogen is suppressed under high moisture conditions (Zhang and Pfender 1992). The pathogen requires a water potential of $\geq$ -0.5 MPa (= -5 bars) to form maximum numbers of pseudothecia, and no pseudothecia are formed below a water potential of -2.5 MPa (=-25 bars) (Pfender et al. 1988). The pseudothecia enlarge during the cropping season and asci are formed from early winter to spring.

Conidia produced on the old lesions are also dispersed by the wind. Schilder and Bergstrom (1992) reported that most conidia were dispersed within 3 m of the initial inoculum. They were also recovered within 25 m of the inoculum source and a few conidia were sometimes recovered up to 100 m away from the inoculum. Increasing levels of air-borne conidia have been observed during dry afternoons for several days after rain. The percentage of liberation of conidia increases with increasing windspeed under high RH, while small amounts of conidia are released under conditions of low RH (Francl 1998; Platt and Morrall 1980b).

Germ tube growth and production of appressoria are positively correlated with lengthening of the post-inoculation wet period and higher temperatures (Francl 1998, Hosford et al. 1987). These environmental factors, especially water potential and wet period, also have a great effect on the formation of papillae (Hosford et al. 1987). Conidial germination and number of germ tubes are not correlated with host resistance (Hosford et al. 1987; Larez et al. 1986).

Virulence of the pathogen to hosts is maximized between 20 to 28 °C (Luz and Bergstrom 1986) and suppressed above 27 °C (Lamari and Bernier 1994; Orolaza et al. 1995). High temperatures, particularly over 27 to 30 °C, are believed to affect the sensitivity of the host to pathogen-produced toxins (Ptr ToxA and Ptr ToxB) and could be an important factor affecting spread of the disease in the field (Lamari and Bernier 1994; Sone et al. 1994). Understanding the epidemiology of the disease is important for estimating yield losses and applying appropriate management strategies (De Wolf et al. 1998). Forecasting systems have been developed for tan spot, and are based on artificial neural networks (De Wolf and Francl 1997; Gregoire 2005). Infection periods on susceptible wheat genotypes were identified by correlating disease incidence with the crop growth stage and 24 h summaries of temperature, relative humidity, precipitation, wind direction, and leaf wetness duration (De Wolf and Francl 1997). One forecasting system also predicted disease development based on wet period duration, precipitation and relative humidity, with an accuracy of 82% (Gregoire 2005). Forecasting systems are important for predicting disease outbreaks and yield losses, but the severity of tan spot of wheat can be difficult to assess since

the disease is often associated with other foliar diseases in the field (De Wolf et al. 1998).

1.1.4 Histological and Cytological Studies

According to Larez et al. (1986), both ascospores and conidia of *P. tritici-repentis* land on wheat plants and germinate, forming appressoria on the germ tubes. Germ tubes are produced from polar and intercalary cells of the conidia. Appressoria are club-shaped or round, are formed at the end of the germ tubes and are generally localized on the epidermal cell junctures (Larez et al. 1986). Penetration pegs from the appressoria enter into and grow in the host epidermal cells. Sometimes, the pathogen also penetrates and infects through stomata, without forming appressoria. Papillae are formed by the host around the infecting hyphae beneath the appressoria. The formation of papillae is associated with disease resistance and is common in Poaceae (Sherwood and Vance 1980). After penetration, secondary hyphae are formed from vesicles and develop intercellularly in the mesophyll (Lamari and Bernier 1989b; Larez et al. 1986). Conidial germination, appressorium formation, penetration of epidermal cells, formation of intracellular vesicles, and growth of intracellular hyphae in epidermal cells occurs within 12 hours after inoculation, while growth of hyphae in the mesophyll follows in the next 12 hours (Loughman and Deverall 1986). Therefore, penetration and infection generally occur within the first 24 hours after inoculation (Dushnicky et al. 1996; Larez et al. 1986). *Pyrenophora tritici-repentis* penetrates wheat epidermal cells, regardless of whether it is on a susceptible or

resistant cultivar (Heath 1972; Skipp and Deverall 1972). Loughman and Deverall (1986) reported that intercellular hyphae developed more rapidly in the mesophyll of susceptible versus resistant wheat. This could be related to the speed of symptom development, which differs between susceptible and resistant wheat genotypes. Development of secondary hyphae on resistant wheat was also limited to infection of the epidermal cells and/or localized areas of the mesophyll at the infection site (Dushnicky et al. 1998a; 1998b; Lamari and Bernier 1989b). The spread of intercellular hyphae is restricted by the larger mid-veins of the wheat leaves (Dushnicky et al. 1998b). Dushnicky et al. (1998b), in a histological study, identified the presence of lignin or lignin-like material in the intercellular space of the mesophyll, and concluded that the deposition of lignin was related to the resistance response of the host.

1.1.5 Symptoms

The pathogen, *Pyrenophora tritici-repentis* can infect the whole plant including leaves, kernels (seed), and stem tissue. On susceptible wheat leaves, the first symptoms appear on the lower leaves during spring and summer. The fungus produces tan colored, oval lesions, often with a small dark brown spot at the centre. Shortly thereafter, the lesions enlarge and may develop chlorotic halos. As more lesions develop, the chlorotic areas, and sometimes the necrotic areas, can coalesce on severely infected leaves. Pinhead-size pseudothecia are formed on infested straw, but not on the leaves during the growing season (Menzies and Gilbert 2003; Shabeer and

Bockus 1988; Wiese 1987). Several rating systems for evaluation of tan spot severity have been proposed, most of which are based on lesion type (Gilchrist et al. 1984; Hosford 1971) or the percentage of total leaf area covered by lesions (Luz and Bergstrom 1980; Nagle et al.1982; Raymond et al. 1985). Lamari and Bernier (1989a) developed a 1-5 rating system, based on lesion type, where: 1 = small dark brown to black spots without any surrounding chlorosis or tan necrosis, 2 = small dark brown to black spots with very little chlorosis or tan necrosis, 3 = small dark brown to black spots completely surrounded by a distinct chlorotic or tan necrotic ring, with the lesions generally not coalescing, 4 = small dark brown to black spots completely surrounded with tan necrotic or chlorotic zones, with some of the lesions coalescing, and 5 = most lesions consisting of coalescing chlorotic or tan necrotic zones, with the dark brown or black centers not always distinguishable.

The infection of kernels is characterized by a pink and/or red discoloration, known as red smudge. As lesions develop, dark smudges and black points are observed (Fernandez et al. 1994a; 1994b; 1998). The red smudge symptom in wheat kernels is similar to that caused by fusarium scab (head blight) and bacterial pink seed (Menzies and Gilbert 2003). Infected kernels are commonly found in Kenya and Australia, but rarely in North America (Hosford 1982). However, all wheat cultivars registered in western Canada are susceptible to infection of the kernels (Fernandez et al. 1998).

1.1.6 Pathotypes and Races

Isolates of *P. tritici-repentis* were initially classified into four pathotypes based on their ability to induce chlorotic (chl^+, symptoms produced or chl^-, symptoms not produced) and/or necrotic (nec^+, symptoms produced or nec^-, symptoms not produced) symptoms on specific wheat differential lines and cultivars (Lamari and Bernier 1989b). However, the pathotype classification system allowed for a theoretical maximum of only four groups (pathotype 1: nec^+chl^+, pathotype 2: nec^+chl^-, pathotype 3: nec^-chl^+, and pathotype 4: nec^-chl^-), and could not accommodate cases where the same symptom was induced on different wheat lines/cultivars. This limitation became evident when isolates were identified that belonged to pathotype 3, since they induced chlorosis, but did so on different host genotypes when compared with other pathotype 3 isolates (Lamari et al. 1995). Therefore, a race classification system was proposed, in which isolates were grouped into races based on the reaction of a wheat differential set (Lamari et al. 1995). This system can accommodate any number of races and is limited only by the uniqueness and number of the differential wheat genotypes used (Lamari et al. 1995).

To date, at least eight races of the pathogen have been identified (Lamari and Bernier 1989b; Lamari et al. 1995; 1998; 2003; Strelkov et al. 2002), based on the reaction of three effective differential genotypes. Races 1 to 4 correspond to the original pathotypes 1 to 4 (Lamari et al. 1995), and are predominant in the U.S.A. and Canada (Lamari et al. 1998). Race 5 was first identified in eastern Algeria and produces similar symptoms to race 3 isolates, the difference being its ability to induce

chlorosis in cultivar 'Katepwa' and line 6B662, but not in 6B365 (Lamari et al. 1995). Race 5 isolates have also since been reported from Canada (Lamari et al. 1998; Strelkov et al. 2002) and the U.S.A. (Ali et al. 1999). In Canada, the sole race 5 isolate identified to date (92-171R5) was collected on the Manitoba–Saskatchewan border and is much less aggressive than other race 5 isolates, inducing smaller lesions and lower levels of chlorosis (Strelkov et al. 2002). Race 6 has been isolated from collections made in eastern Algeria and combines the virulence patterns of races 3 and 5 (Strelkov et al. 2002). Race 7 was first collected in Azerbaijan (Caucasus region) and combines the virulence patterns of races 2 and 5. Race 8 was collected in the Fertile Crescent (Syria-Turkey) region, which represents the host center of diversity, and combines the virulence patterns of races 2, 3, and 5 (Lamari et al. 2003). There have been preliminary reports of additional races, but these have not been characterized yet (Ali and Francl 2002; Ali et al. 2003; Manning et al. 2002; Martinez et al. 2004).

1.1.7 Host Range

Pyrenophora tritici-repentis was first identified on couch grass (*Agropyron repens*). Since then, the host range of the pathogen has been reported to include at least 33 cereal and grass species including durum and spring wheat (Hosford 1982; Krupinsky 1982; 1992). The species has the widest host range of all the species in the *Drechslera* and *Pyrenophora* genera (Shoemaker 1962). According to Hosford (1971), *Agropyron desertorum*, *A. intermedium* (intermediate wheatgrass), *A. repens*, *A.*

smithii (western wheatgrass), *Bromus inermis* (smooth bromegrass), *Triticum aestivum* (spring wheat), *T. araraticum*, *T. carthlicum*, *T. dicoccoides*, *T. durum*, *T. macha*, *T. orientale*, *T. palecolchicum*, *T. pyramicale*, and *Secale cereale* (rye) were found to be highly susceptible to the pathogen. Additionally, the pathogen was also reported to infect *Elymus angustus* (altai wildrye) and *E. giganteus* (mammoth wildrye) (Krupinsky 1992). In contrast, *Avena sativa* (oats), *Elymus junceus* (Russian wildrye), *Linum usitatissimum*, *Medicago sativa*, *Hodeum vulgare* (barley), and *T. turanicum* were found to be resistant to *P. tritici-repentis*. Shoemaker (1962) reported that other hosts of the pathogen included: *Beckmannia syzigachne* (American sloughgrass), *Calamovilfa longifolia* (prairie sandreed), *Phalaris arundinacea* (reed canarygrass), *Leersia oryzoides* (cutgrass), a *Triticum* × *Aprogyron* hybrid, and several other species. Disease symptoms on smooth bromegrass were less severe than on wheat, even though *P. tritici-repentis* isolates collected from bromegrass were used for inoculation (Krupinsky 1987). Non-cereal grass hosts provide overwintering habitat for *P. tritici-repentis*, allowing it to survive in the absence of wheat (Krupinsky 1992).

1.2 Host-Specific Toxins (HSTs)

Host-specific toxins (host-selective toxins, HSTs) are produced by at least 16 species of fungi (Ciuffetti et al. 1997) and serve as pathogenicity factors (Yoder 1980), since they are required for disease development by the pathogen. Most host-specific toxins are secondary metabolites with diverse structures and low molecular masses

(Walton 1996). For instance, AM-toxin, a HST produced by the apple pathogen *Alternaria mali,* has been extensively studied and found to have the molecular formula $C_{23}H_{31}N_3O_6$ (AM toxin-I) or $C_{22}H_{29}N_3O_5$ (AM toxin-II) (Kono et al. 1981). The toxin causes necrosis at concentrations of 0.2 ng/ml in susceptible hosts, or 1.0 µg/ml in resistant hosts. Similarly, AK-toxin, which is produced by *Alternatia kikuchiana* on pears (Hiroe et al. 1958; Tanaka 1933), has a molecular formula of $C_7H_4O_6$ (Sugiyama et al. 1965; 1966a; 1966b). HC-toxin (Scheffer and Ullstrup 1965), which is produced by *Helminthosporium carbonum* on maize, is a cyclic tetrapeptide with the structure: cyclo(D-Pro-L-Ala-D-Ala-L-Aeo) (Walton et al. 1982).

In the case of *P. tritici-repentis*, the eight races of the pathogen that have been identified produce at least three host-specific toxins, termed Ptr ToxA, Ptr ToxB, and Ptr ToxC (Ballance et al.1989; Effertz et al. 2002; Strelkov et al. 1999; Tomás and Bockus 1987). Two of these toxins, Ptr ToxA and Ptr ToxB, are ribosomally-synthesized proteins. The hexaploid wheat cultivar 'Glenlea' carries the gene controlling sensitivity to Ptr ToxA, which is produced by races 1, 2, 7, and 8. Wheat line 6B662 carries the gene controlling sensitivity to Ptr ToxB, produced by races 5, 6, 7, and 8, and line 6B365 carries the gene controlling sensitivity to Ptr ToxC, produced by races 1, 3, 6, and 8 (Lamari et al. 2003). In each case, wheat sensitivity to the toxin and the susceptibility to the fungus are controlled by the same genetic locus (Gamba et al. 1998). In addition, there is preliminary evidence of the production of additional pathogenicity factors or toxins by *P. tritici-repentis* (Ali et al. 2002;

Gamba et al. 1998; Manning et al. 2002). Race 4 isolates are not known to produce any toxins.

1.2.1 Ptr ToxA

Ptr ToxA (formerly Ptr necrosis toxin, [Ciuffetti et al. 1998]) was the first proteinaceous cultivar-specific toxin to be identified from *P. tritici-repentis*, and induces necrosis on sensitive hosts (Tomás and Bockus 1987). Ptr ToxA has been purified and characterized by several research groups (Ballance et al. 1989; Tomás et al. 1990; Tuori et al. 1995; Zhang et al. 1997). All groups isolated a 13-14 kDa heat stable, water-soluble protein. Ballance et al. (1989) reported that the toxin, which they purified from race 2 isolate 86-124, was a heat-stable protein with a mass of 13.9 kDa and an average minimum active concentration of 0.2 nM. Tomás et al. (1990) purified the toxin from isolate Pt-1c, and described it as a heat stable protein with a 14.7 kDa molecular mass and an average minimum active concentration of 90 nM. However, the masses reported by Ballance et al. (1989) and Tomás et al. (1990) were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Using mass spectrometry, Zhang et al. (1997) reported a toxin protein of 13.2 kDa mass from isolate 86-124. Tuori et al. (1995) also reported a heat stable, necrosis-inducing protein of 13.2 kDa mass with an average minimum active concentration of 60 nM, which they obtained from isolate Pt-1c. Comparison of amino acid composition, mass, and other properties strongly suggested that all researchers were dealing with the same toxin, which was named Ptr ToxA in an effort to standardize nomenclature in

the tan spot pathosystem (Ciuffetti et al. 1998). The gene encoding Ptr ToxA, termed *ToxA*, has been cloned and sequenced by several independent research groups (Ballance et al. 1996; Ciuffetti et al. 1997).

ToxA encodes a pre-pro-protein (Ballance et al. 1996, Ciuffetti et al. 1997). The protein consists of a 4.3 kDa anionic N-domain and a 13.2 kDa C-domain. The C-domain has two-cysteine residues that affect protein structure and function (Tuori et al. 2000). Tuori et al. (2000) reported that a mutation of threonine 137, which is N-terminal to an RGD (Arginine-Glycine-Aspartic Acid tripeptide) motif located at arginine 140, decreased toxin activity. Additionally, the glycine and aspartic acid residues of the RGD motif are crucial for toxin activity (Meinhardt et al. 2002). More recently, multiple motifs, including two CK2 phosphorylation-like motifs and a vitronectin-like motif, were found and considered to be associated with Ptr ToxA activity (Manning et al 2004). Manning and Ciuffetti (2005) used green fluorescent protein-tagged Ptr ToxA to show that the toxin is internalized, in sensitive wheat genotypes only, to cytoplasmic compartments and chloroplasts of the host cells. However, intracellular expression of Ptr ToxA in both sensitive and insensitive genotypes resulted in cell death, suggesting that its internal site of action is present in both cases (Manning and Ciuffetti 2005). Therefore, the Ptr ToxA sensitivity gene is likely related to protein import.

According to Lamari and Bernier (1994), wheat plants that are infiltrated with Ptr ToxA become insensitive to the toxin at 27 °C, but toxin sensitivity is recovered after the plants are moved to 22 °C. Given the heat stability of Ptr ToxA (Ballance et

al. 1989), Lamari and Bernier (1994) suggested that this insensitivity at higher temperatures may result from conformational changes in the host target or receptor, which prevent proper binding of the toxin molecule. Sensitivity to Ptr ToxA in the host is controlled by a single recessive gene (Lamari and Bernier 1991). The gene, termed *tsn1*, is located on the long arm of chromosome 5BL in common and durum wheat and confers sensitivity to Ptr ToxA (Gamba and Lamari 1998). However, Friesen et al. (2003) reported that *tsn1* accounted for only 24.4 % of the phenotypic variation in resistance to the disease. They found that four mutant wheat lines that were insensitive to Ptr ToxA were not resistant to the pathogen, although the disease did develop more slowly than in the wild-type (Friesen et al. 2003). Nevertheless, the transfer of the *ToxA* gene into a non-toxin producing isolate resulted in toxin production and pathogenicity (Ciuffetti et al. 1997).

Interestingly, a proteinaceous host-specific toxin produced by *Stagonospora nodorum*, designated Sn ToxA, has been recently identified and found to be homologous to Ptr ToxA (Friesen et al. 2006). *Stagonospora nodorum* often occurs as part of a cereal leaf spotting complex with *P. tritici-repentis*, and it is hypothesized that the *ToxA* gene may have been transferred from one pathogen to the other (Friesen et al. 2006).

1.2.2 Ptr ToxB

Ptr ToxB (syn. Ptr chlorosis toxin [Ciuffetti et al. 1998]) induces chlorosis symptoms in sensitive wheat lines/cultivars. Like Ptr ToxA, this toxin is also a heat

stable protein, maintaining full toxic activity after 1 hour at 55 °C (Strelkov et al. 1999). The molecular mass of Ptr ToxB was determined to be 6.61 kDa by mass spectrometry and the toxin was found to have a very high level of toxic activity, producing chlorosis on susceptible cultivars at concentrations as low as 14 nM (Strelkov et al. 1999). Although Ptr ToxB-producing isolates are rare in Canada (Strelkov et al. 2002; Strelkov and Lamari 2003), large areas of the prairies have been planted to Ptr ToxB-sensitive wheat cultivars (Lamari et al. 2005). Therefore, a potential threat exists if these isolates were to become more common.

Ptr ToxB is encoded by a multiple copy gene, termed *ToxB*, which was identified in races 5, 6, 7, and 8 of *P. tritici-repentis* (Lamari et al. 2003; Martinez et al. 2001). The *ToxB* gene codes for an 87 amino acid residue protein, including a 23 residue signal peptide (Martinez et al. 2001; Strelkov 2002). Homologs of the *ToxB* gene were also found in races 3 and 4, even though these are lacking Ptr ToxB activity (Strelkov and Lamari 2003; Martinez et al. 2004; Strelkov et al. 2006). The *ToxB* gene is absent in isolates from races 1 and 2 (Strelkov 2002; Strelkov et al. 2006). In race 4, the *ToxB* homolog (referred to as *toxb* by Martinez et al. 2004) occurs as a single copy, and independent analysis of this gene from two different isolates indicated that it possesses only 86% similarity to 'wild-type' *ToxB* from race 5 isolates (Martinez et al. 2004; Strelkov et al. 2006). However, the role of *ToxB* homologs in race 4 isolates is not clear, since Ptr ToxB does not appear to control vital biologic functions in *P. tritici-repentis* aside from its role in pathogenesis (Strelkov and Lamari 2003).

Friesen and Faris (2004) mapped the Ptr ToxB insensitivity gene, *tsc2*, to the short arm of chromosome 2B in the wheat host. The gene is associated with resistance to race 5 of *P. tritici-repentis* and accounted for 69 % of phenotypic variation in resistance to race 5 isolate DW5 (Friesen and Faris 2004). According to Strelkov et al. (1998), the toxin appears to inhibit photosynthesis, likely as a result of the active oxygen-mediated photo-oxidation of chlorophyll. The toxin does not directly affect plant water and carbon dioxide exchange with the atmosphere (Strelkov 2002). The development of the chlorosis symptom is also suppressed at 27 °C, as was observed with the necrosis symptom induced by Ptr ToxA (Lamari and Bernier 1994). This suppression may also result from a conformational change in the toxin target or receptor at higher temperatures (Lamari and Bernier 1994).

1.2.3 Ptr ToxC and Other HSTs

Ptr ToxC, which induces chlorosis on wheat line 6B365 but not on 6B662, is clearly a different toxin from Ptr ToxB. Ptr ToxC was partially purified from a race 1 isolate of *P. tritici-repentis* and found to be a nonionic, polar, low-molecular-weight molecule, but not proteinaceous in nature (Effertz et al. 2002). However, this toxin remains to be fully characterized. Resistance in wheat to race 3 isolates was found to be predominantly controlled by *Qtsc.ndsu-1A* at a locus on chromosome 4AL (Effertz et al. 2001), the same chromosome on which resistance to race 1 is found (Faris et al. 1997; 1999). The gene conferring sensitivity to Ptr ToxC, termed *tsc1*, was mapped to the same *Qtsc.ndsu-1A* locus (Effertz et al. 2002), further supporting earlier studies

indicating that sensitivity to this toxin and susceptibility to isolates that produce it are controlled by the same genetic locus (Gamba et al. 1998).

In addition to Ptr ToxA, Ptr ToxB and Ptr ToxC, there are preliminary reports of the existence (Ali et al. 2002; Manning et al. 2002), or likely existence (Gamba et al. 1998), of other host-specific toxins produced by *P. tritici-repentis*. These reports require confirmation and await further characterization. Furthermore, *P. tritici-repentis* also produces another class of necrosis-inducing toxins, called triticones, which are not host-specific. The triticones are spirocyclic lactams and are therefore distinct from Ptr ToxA (Hallock et al. 1993).

1.3 Tan Spot Disease Management

The main ways to control tan spot of wheat include chemical treatment, use of appropriate cultural practices, and the adoption of resistant cultivars (De Wolf et al. 1998). The fact that the tan spot pathogen often occurs as part of a complex of necrotrophic foliar diseases, and that it can also occur with biotrophic fungi such as the powdery mildews and rusts, makes development of effective management strategies a challenge (De Wolf et al. 1998; Luz and Bergstrom 1987; Pfender et al. 1993). Nevertheless, a number of control methods have been evaluated and may be routinely used.

1.3.1 Fungicides

Fungicides effective against *P. tritici-repentis* include dithiocarbamate- and triazol-based formulations (Mehta and Gaudêncio 1991). Tilt (propiconazole) and other systemic fungicides are registered for wheat, barley, and disease control in other gramineous crops. The protectant fungicide Mancozeb (dithiocarbamate-based fungicide) is effective against tan spot and must be sprayed before the appearance of symptoms in the field (McMullen and Francl 1993). The dithiocarbamate-based fungicide Maneb, plus a zinc ion fungicide, reduced leaf symptoms by an average rating of 1.0 and increased wheat yield by an average of 11.2 % (Hosford and Busch 1974). Hosford and Busch (1974) also noted that other fungicides that were registered against Septoria leaf diseases could be used against tan spot of wheat. Currently, several foliar fungicides including some strobilurins are registered for use against tan spot in wheat (Anon. 2006). For seed treatments, an organo-mercurial dust dressing was identified as effective for disease control (Duff 1954); however, nothing is currently registered for use against seed-borne tan spot (Anon. 2006).

1.3.2 Biological Control

Recent surveys showed that wheat growers are interested in using biological control products, and the availability of such products may be increasing in the future (Perelló et al. 2003). Indeed, some biological antagonists of *P. tritici-repentis* have already been reported. To date, *Laetisaria roseipellis*, for treatment of stubble residue (Pfender et al.1993), *Paenibacillus macerans* and *Pseudomonas putida* for treatment

of kernels (Luz et al. 1998), and *Trichoderma* spp. for protection of the whole plant (Perelló et al. 2003) have been studied as disease control agents and identified as effective against the pathogen in field tests. In general, some species in the genus *Trichoderma* are well known for their antagonistic properties against other fungi on commercial crops. Interestingly, *Cochliobolus sativus* and *Limonomyces roseipellis* suppress the development of tan spot in the field, while they have been known to cause other diseases on wheat (Luz and Bergstrom 1987; Pfender et al. 1993). These fungi affect conidial germination, appressorium formation, and germ tube elongation by *P. tritici-repentis* on the leaf surface (Luz and Bergstrom 1987). The combination of chemical fungicides and biological antagonists may help to enhance disease control.

1.3.3. Cultural Control

Crop rotation and burying of wheat residue by tillage can reduce the amount of pathogen inoculum, and consequently reduce disease severity in the next season. Non-host crops such as corn, mustard, flax, and soybean can help in rotation, but oat and barley are not immune (Menzies and Gilbert 2003). In the Canadian prairies, tillage practices vary widely. Most wheat producers in this region traditionally relied on mechanical tillage for weed control and seedbed preparation. However, in recent years, wheat producers have changed their farming methods to combinations of tillage and herbicide application (Fernandez et al 1998). Infected crop residues can be considered as a source of primary inoculum for *P. tritici-repentis*. Conservation tillage

helps control soil erosion, which is caused by the loss of moisture in soil (Fernandez et al 1998), but can lead to increased amounts of inoculum on the soil surface (McMullen and Francl 1993; Zhang and Pfender 1992). Burning of wheat stubble helps to reduce the levels of primary inoculum, resulting in decreased disease severity on the primary leaves, although it does not control it completely. Unfortunately, stubble burning will also reduce soil organic matter and bring about serious wind and rain erosion (Mehta and Gaudêncio 1991). Huber et al. (1987) reported that the application of nitrogen reduced disease severity and increased yield.

1.3.4 Genetic Resistance

The best measure for controlling tan spot of wheat is genetic resistance in the host, since it is most effective and least costly for producers (De Wolf et al. 1998). Unfortunately, all cultivars available in Canada are susceptible to tan spot (Menzies and Gilbert 2003). According to Lamari et al. (2005), wheat cultivars sensitive to Ptr ToxA have been cropped continuously in western Canada since the 1960s. This may explain the susceptibility of Canadian wheat cultivars to *P. tritici-repentis*, as isolates producing Ptr ToxA (i.e. races 1 and 2) are predominant in North America. Furthermore, as noted above, Ptr ToxB-sensitive wheat cultivars were also introduced to western Canada in 1934 and have been planted over large areas. Although most hexaploid wheat cultivars in western Canada are resistant to race 3 and likely insensitive to Ptr ToxC, this race is rare in the region (Lamari et al. 1998; Lamari et al. 2005).

1.4 Research Objectives

Martinez et al. (2004) reported that the homolog of *ToxB* (*toxb*) which they identified in a race 4 isolate of *P. tritici-repentis* from North Dakota was not expressed in mycelia under conditions favoring expression of wild-type *ToxB* in race 5 isolates. Strelkov et al. (2006) confirmed this apparent lack of expression of the gene in mycelia of another race 4 isolate and in a low virulence race 5 isolate from the Canadian prairies, and provided preliminary evidence that the gene was expressed in conidia of these same isolates. The primary objective of the current research was therefore to characterize the expression of the different forms of the *ToxB* gene in virulent, low virulence and avirulent isolates of *P. tritici-repentis*, in conidia and in mycelial culture, as well as to determine the *ToxB* expression pattern on Ptr ToxB-sensitive and insensitive wheat genotypes. Information on the expression of this gene could provide insights into quantitative variation in the virulence of *P. tritici-repentis* isolates, as well as clues as to the role of *ToxB* homologs in avirulent isolates of the fungus. In addition, previous research conducted using mainly necrosis-inducing isolates suggested that the virulence bestowed by the Ptr toxins is superimposed over a basic compatibility between *P. tritici-repentis* and its wheat host (Strelkov and Lamari 2003). However, this issue has not been examined with respect to Ptr ToxB and the Ptr ToxB-producing isolates. Therefore, a secondary objective of the present research was to study the behavior of virulent, low virulence and avirulent isolates of the fungus on Ptr ToxB-sensitive and insensitive wheat genotypes, in order to learn more about the infection process.

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2.0 Quantification of *ToxB* Gene Expression in Virulent and Avirulent Isolates of *Pyrenophora tritici-repentis*, Causal Agent of Tan Spot of Wheat.

2.1 Introduction

Tan spot, caused by the homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.), is an important foliar disease of wheat throughout the major wheat growing regions of the world. The pathogen induces two distinct symptoms on susceptible wheat genotypes: tan necrosis and/or extensive chlorosis. Isolates of *P. tritici-repentis* were initially classified into four pathotypes based on their ability to induce these symptoms on specific wheat differential lines (Lamari and Bernier 1989b). However, the pathotype classification system allowed for a theoretical maximum of only four groups (pathotype 1: $nec^{+}chl^{+}$, pathotype 2: $nec^{+}chl^{-}$, pathotype 3: $nec^{-}chl^{+}$, and pathotype 4: $nec^{-}chl^{-}$) and was later extended to accommodate isolates that induced the same symptom on different wheat lines/cultivars (Lamari et al. 1995). A race classification system was adopted, which was based on the reaction of isolates on individual wheat genotypes (Lamari et al. 1995). This system can accommodate any number of races and is limited only by the uniqueness and number of the differential wheat genotypes used (Lamari et al. 1995). To date, eight races of the pathogen have been identified according to their virulence on three effective wheat differentials, namely line 6B365, line 6B662, and 'Glenlea' (Lamari and Bernier 1989b; Lamari et al. 1995; 1998; 2003; Strelkov et al. 2002).

The development of necrosis and chlorosis symptoms on affected wheat leaves results from the differential production of host-specific toxins (HSTs) by isolates of *P. tritici-repentis*. At least three HSTs, termed Ptr ToxA, Ptr ToxB and Ptr ToxC, are produced, all of which appear to be pathogenicity factors (Effertz et al. 2002; Lamari and Bernier 1989c; Orolaza et al. 1995). In addition, there are preliminary reports of the production of at least two other toxins by the pathogen (Ali et al. 2002; Manning et al. 2002). Ptr ToxA (syn. Ptr toxin, Ptr necrosis toxin, and ToxA [Ciuffetti et al. 1998]) is a 13.2 kDa protein (Ballance et al. 1989; Tomás et al. 1990; Tuori et al. 1995; Zhang et al. 1997) that induces necrosis on sensitive wheat genotypes. Ptr ToxB (syn. Ptr chlorosis toxin [Ciuffetti et al. 1998]) induces chlorosis in sensitive wheat lines/cultivars, and is also a protein with a 6.61 kDa mass (Strelkov et al. 1999). Ptr ToxC, which has only been partially characterized and appears to be a low molecular mass molecule, also induces chlorosis, but not on the same wheat genotypes as Ptr ToxB (Effertz et al. 2002).

The genes encoding Ptr ToxA and Ptr ToxB, termed *ToxA* and *ToxB*, respectively, have been cloned and characterized by several independent research groups (Ballance et al. 1996; Ciuffetti et al. 1997; Strelkov and Lamari 2003; Martinez et al. 2004; Strelkov et al. 2006). However, while *ToxA* is found as a single copy only in isolates of *P. tritici-repentis* that produce Ptr ToxA (Ballance et al. 1996; Ciuffetti et al. 1997; Lamari et al. 2003), *ToxB* is a multiple copy gene, forms of which are also found in isolates that lack Ptr ToxB toxin activity (Strelkov and Lamari 2003; Martinez et al. 2004; Strelkov et al. 2006). Thus, while the *ToxB* gene has been

identified in isolates representing races 5, 6, 7 and 8 of *P. tritici-repentis*, all of which produce chlorosis on the Ptr ToxB-sensitive wheat line 6B662, homologs of the gene have also been found in races 3 and 4 isolates, which possess no toxin activity (Lamari et al. 2003; Martinez et al. 2004; Strelkov et al. 2006). In race 4, the *ToxB* homolog (referred to as *toxb* by Martinez et al. 2004) occurs as a single copy, and independent analysis of this gene from two different isolates indicated that it possesses only 86% similarity to 'wild-type' *ToxB* from race 5 isolates (Martinez et al. 2004; Strelkov and Lamari 2003). However, the role of *ToxB* homologs in race 4 isolates is not clear, since Ptr ToxB does not appear to control vital biologic functions in *P. tritici-repentis*, aside from its role in pathogenesis (Strelkov and Lamari 2003).

Martinez et al. (2004) reported that the form of *ToxB* (*toxb*) that they identified in a race 4 isolate from North Dakota was not expressed in mycelia under conditions favoring expression of wild-type *ToxB* in race 5 isolates. Strelkov et al. (2006) confirmed this apparent lack of expression of the gene in mycelia of a race 4 isolate from the Canadian prairies, and provided preliminary evidence that the gene was expressed in conidia of the same isolate. Similarly, Strelkov et al. (2006) also reported that a form of *ToxB* was expressed in conidia, but not 3 to 9 day-old mycelia, of a Canadian race 5 isolate that had previously been shown to be of low-virulence (Strelkov et al. 2002). However, there are no data available on expression of *ToxB* and its homologs in planta, and expression of the different forms of the gene has not been quantified or compared under any conditions. Information on the activity of the forms of *ToxB* in avirulent and low virulence isolates of the pathogen may provide

clues as to the role of the gene in these isolates. Therefore, the objective of the present study was to characterize the expression patterns of the different forms of the *ToxB* gene in virulent, low virulence and avirulent isolates of *P. tritici-repentis* in conidia, in culture and in planta, using reverse-transcriptase (RT)-PCR and quantitative real-time (qRT)-PCR, which is a specific and sensitive method for reproducible quantification of mRNA (Bustin 2000).

2.2 Materials and Methods

2.2.1 Fungal Isolates

Three isolates of *P. tritici-repentis*, representing races 4 and 5 of the pathogen, were used in this study. Race 4 isolate 90-2 was obtained from the Canadian prairies and is an avirulent isolate (Lamari et al. 1998) lacking Ptr ToxB activity. Race 5 isolates Alg3-24 and 92-171R5 were collected in eastern Algeria and the Saskatchewan-Manitoba border (Lamari et al. 1995; Strelkov et al. 2002), respectively, and while both possess Ptr ToxB activity, that activity is much weaker in 92-171R5 (Strelkov et al. 2002). Isolates were grown in Petri dishes on V8-potato dextrose agar (PDA) and conidial inoculum was produced as previously described (Lamari and Bernier 1989c). To produce mycelia in liquid culture, five plugs (1 cm in diameter) were cut from 4-5 cm diameter colonies of the fungus and transferred to Erlenmeyer flasks containing 150 ml of Fries medium(amended with 0.1 % yeast extract), and incubated for 5, 10, 15, or 20 days in the dark at room temperature with no agitation.

2.2.2 Plant Material and Inoculation

Two hexaploid wheat genotypes, 6B662 and 'Erik,' were inoculated with *P. tritici-repentis*. Line 6B662 is Ptr ToxB-sensitive, resistant to race 4 and susceptible to race 5, while 'Erik' is Ptr ToxB-insensitive and resistant to both races. Five to six evenly spaced seeds were sown in 10 cm plastic pots filled with Metro-Mix 220 soil (W.R.Grace and Co., Ajax, ON), and seedlings were maintained in a greenhouse at 20°C/18°C (day/night) with a 16 hour photoperiod at 250 μmol $m^{-2}s^{-1}$ (natural light supplemented with artificial lighting) until inoculation at the 2-3 leaf stage. The seedlings were inoculated with a suspension of 3500 conidia per ml to which 10 drops of Tween 20 (polyoxyethylene sorbitol monolaurate) per liter were added, using a sprayer connected to an air line. Leaves were sprayed until run-off. Negative controls were inoculated with sterile distilled H_2O containing the same concentration of Tween 20. Immediately following inoculation, the plants were placed under darkness in a misting chamber (relative humidity ≥ 95%) for a 24 h period, with continuous wetness provided by an ultrasonic humidifier. After incubation under high humidity, the plants were transferred to a growth chamber and kept at 20 °C/18 °C (day/night) with a 16-hour photoperiod (180 μmol $m^{-2}s^{-1}$) and 60 % relative humidity. Tissue from the middle of the second leaves was sampled for analysis at 0, 12, 24, 48, 72, 120, 144, and 168 h after inoculation. To confirm infection by *P. tritici-repentis*, single conidium of the fungus was re-isolated as per Lamari et al. (1995) from leaves harvested 168 h after inoculation. Experiments were independently repeated a total of 3 times, with 3 to 4 technical replicates at each time point.

2.2.3. Disease Development

To compare the development of chlorotic symptoms in the two wheat hosts after inoculation with the various isolates, the percentage of the total foliar area covered by lesions on each genotype was measured using image analysis software (Lamari 2002). Leaves were scanned on a flatbed scanner (Epson, Toronto ON) immediately after harvest and the total leaf and lesion areas determined using the image analysis software.

2.2.4 Chlorophyll/Carotenoid Concentration

To estimate chlorophyll and carotenoid concentration in inoculated wheat leaves, 3 to 4 leaf segments (total weight 40 mg) were randomly chosen in each treatment and processed as previously described (Strelkov et al. 1998). Briefly, leaves were cut, weighed, and ground in a mortar and pestle in the presence of liquid nitrogen. Homogenates were extracted 3× with 1 ml of 80 % acetone (v/v), the extracts were combined, and the total volume was adjusted to 4 mL with 80% acetone (v/v), corresponding to a final concentration of 10 mg leaf tissue per 1 mL of acetone (Witham et al. 1971). The optical density of the extracts was measured at 470, 647, and 663 nm with a spectrophotometer (Hewlett Packard, Mississauga, ON) and the concentration of chlorophyll and carotenoid pigments was calculated using the equations of Lichtenthaler (1987).

2.2.5 RNA Extraction

For total RNA extraction, conidia and 5, 10, 15 and 20 day-old mycelia of *P. tritici-repentis* were harvested as previously described (Strelkov et al. 2002), lyophilized and stored at -80°C until processing. Leaf samples were flash frozen in liquid nitrogen at the time of harvest and processed immediately. Total RNA was extracted from leaf and fungal samples using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON), following the manufacturer's protocol. After extraction, RNA samples were treated with RNase-free DNase I (Ambion, Austin, TX) to remove any DNA contamination. The total RNA concentration of each sample was quantified spectrophotometrically using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and confirmed by comparing band intensities on 1.2 % denaturing agarose gels using a gel documentation system (Gel Doc 1000, Bio-Rad, Mississauga, ON).

2.2.6 Conventional Reverse Transcriptase (RT)-PCR

For RT-PCR, 1 µg of total RNA from each sample was reverse-transcribed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Burlington, ON) using an oligo$(dT)_{12-18}$ primer. To ensure that all RT-PCR products obtained were amplified from total RNA and not from any residual genomic DNA contamination, control reactions to which no reverse transcriptase was added were also included for each treatment. The *ToxB* cDNA was amplified using the *ToxB*-specific primers TOXBF3

(5'-AAAAAGCAGGCTCCATGCTACTTGCTGTGGCT-3') and TOXBR3 (5'-AGAAAGCTGGGTCTATACCTAATGTAGGGCTAC-3') (Strelkov et al. 2006), in a 25 µl volume containing 2.5 µl of 10× PCR buffer, 0.75 µl of 50mM $MgCl_2$, 0.5 µl of 10mM dNTP, 0.5 µl each of 10 µM forward and reverse primers, 0.2 µl of Platinum® *Taq* DNA Polymerase (0.5 unit/µl), and 2 µl of cDNA from the first-strand reaction. The amplification cycle consisted of an initial heat denaturation step of 4 min at 94°C, followed by 40 cycles of 45 sec at 94°C, 45 sec at 54°C, 45 sec at 72°C, and a final extension of 7 min at 72°C. The presence or absence of amplicons was confirmed by running samples on 1.2% agarose gels.

2.2.7 Endogenous Control for Quantitative Real Time-PCR (qRT-PCR)

The actin housekeeping gene was selected as an endogenous control to normalize *ToxB* expression data obtained using qRT-PCR (Suzuki et al. 2000; Kim et al. 2003; Yan and Liou 2006). Since no actin sequence information was available for *P. tritici-repentis*, the gene was sequenced from isolates Alg 3-24, 92-171R5 and 90-2 of the fungus. Genomic DNA was extracted from 10 day-old mycelial mats of each fungal isolate using a standard CTAB method (DePriest et al. 2005) and subjected to PCR using the universal actin forward primer Act-1 (5'-TGGGACGATATGGAIAAIATCTGGCA-3') and the reverse primer Act-5ra (5'-TTAGAAGCACTTNCGGTG-3') (Voigt and Wostemeyer 2000; Voigt et al. 2005). Reaction conditions were as previously reported (Voigt and Wostemeyer 2000; Voigt et al. 2005), except that the temperature profile was slightly modified

and consisted of 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 1 min at 52°C, 1 min at 72°C, and a final extension of 7 min at 72°C. The PCR products were visualized on agarose gels and subsequently purified using the QIAquick PCR Purification Kit (Qiagen, Mississauga, ON) as per manufacturer's instructions. DNA sequencing was performed on a CEQ2000XL DNA Analysis System (Beckman Coulter, Mississauga, ON) in the Department of Biochemistry, University of Alberta.

2.2.8 Preparation of TaqMan Probe and Primers

All primers and probes used in qRT-PCR were designed using Primer Express Software (PE-Applied Biosystems, Foster City, CA). The primers and probe for quantifying *ToxB* expression were based on conserved regions of the gene from *P. tritici-repentis* isolates Alg 3-24, 92-171R5 and 90-2 (GenBank accession nos. AF483831, AF483834 and AF483832, respectively). Forward primer ToxBqF2 (5'-CATGCTACTTGCTGTGGCTATCC-3') and reverse primer ToxBqR2 (5'-GGACACAGCCAGTCGCAAT-3') were utilized in conjunction with the TaqMan MGB (minor groove-binding) probe ToxBq2 (5'-CTTGTTTCGGCCAACTG-3') to amplify a 104 bp product from the *ToxB* ORF. The primers and probe for the endogenous control were based on the actin gene sequences obtained from isolates Alg3-24, 92-171R5 and 90-2 (see above) and included the forward primer ACTqF1 (5'-CTACGAGCTTCCCGACGGT-3'), reverse primer ACTqR1 (5'-TCTGGAGCACGGAAACGC-3'), and the TaqMan MGB probe ACTq1 (5'-AAGTCATACCCATTGGC-3'). The probe and primers,

which amplified an 60 bp product, were designed to bind to regions of the actin sequence specific to *P. tritici-repentis* and not wheat, so that they could be used to quantify expression of the gene by the fungus in the host. Thus, only 15 of 19 bases were common to ACTqF1 and the wheat actin sequence, 11 of 18 bases were common to ACTqR1 and wheat, and 14 of 17 bases were common to the ACTq1 probe and wheat. The ToxBq2 probe was labeled with the fluorescent reporter dye FAM™ at its 5'-end, while the ACTq1 probe was labeled with VIC® (Applied Biosystems, Foster City, CA).

2.2.9 Quantitative Real Time-PCR (qRT-PCR)

Complementary cDNA was produced as described above for the reverse transcriptase step of the conventional RT-PCR. Quantitative real time-PCR amplifications were conducted as uniplex reactions in Micro Amp 96 well reaction plates in an ABI PRISM® 7700 Sequence Detector (Applied Biosystems). Reaction mixtures consisted of 10 µl TaqMan Universal Master Mix (2×) (Applied Biosystems), 0.225 µl each of 100µM forward and reverse primers, 0.05 µl fluorogenic probe (100 µM), and either 20 ng cDNA in the in vitro and conidial studies, or 50 ng cDNA in the in planta studies. Volumes were adjusted to 20 µl with DEPC-treated water and reactions were conducted under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Fluorescent emissions were recorded in real time at 518 nm and 554 nm for the FAM and VIC reporter dyes, respectively, and data were collected using Sequence

Detector V.1.7 software (Applied Biosystems). Controls which had not been subjected to treatment with reverse-transcriptase or to which no template had been added were included in all qRT-PCR assays, as were controls containing only host cDNA. As noted above, the actin gene was used as the endogenous control for normalizing *ToxB* transcript profiles in all experiments. When examining *ToxB* expression in vitro or in conidia, the data were calibrated relative to *ToxB* transcript levels in 5 day-old mycelia of isolate 90-2, according to the comparative C_T method for relative quantification of gene expression (Livak and Schmittgen 2001). In the in planta studies, qRT-PCR data from each isolate/host combination were calibrated relative to *ToxB* levels at 0 h for isolate 90-2 on the same host, following the comparative C_T method (Livak and Schmittgen 2001). After qRT-PCR, the amplicons obtained were visualized on 3 % agarose gels.

2.3 Results

2.3.1 Disease Development

As expected, chlorosis symptoms were induced by race 5 isolates Alg3-24 and 92-171R5 on the Ptr ToxB-sensitive wheat genotype 6B662 (Fig. 2-1). Chlorotic lesions begun to appear as few as 48 h after inoculation with isolate Alg3-24, or 72 to 96 h after inoculation with 92-171R5 (Fig. 2-2A). In response to Alg3-24, chlorosis spread throughout the affected leaves, and by 168 h after inoculation, approximately 50% of the total leaf area was chlorotic. In contrast, isolate 92-171R5 induced weaker symptoms, and only 13% of the total leaf area was chlorotic in response to

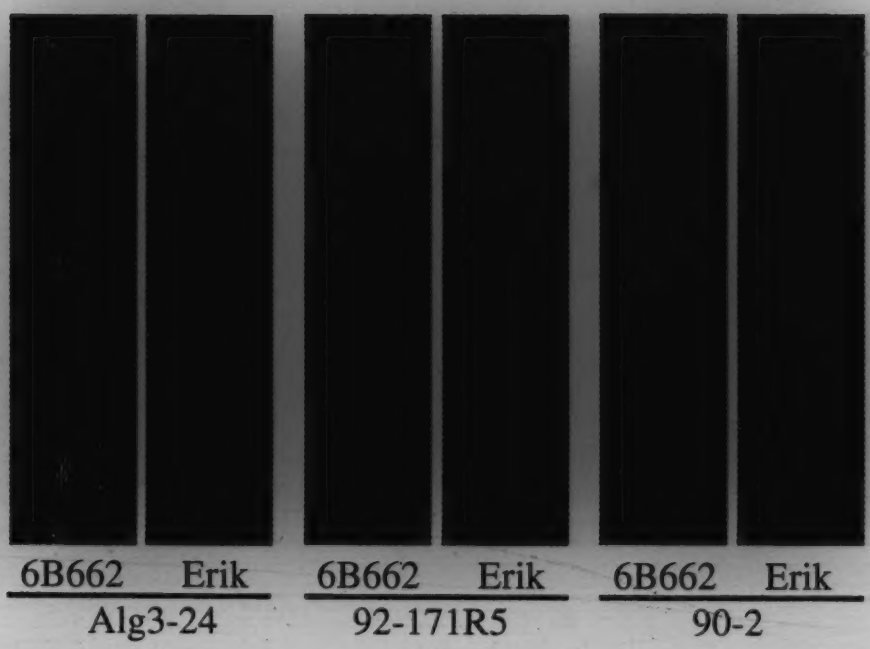

Figure 2-1. Reaction of wheat line 6B662 and cultivar 'Erik' to inoculation with race 5 isolates Alg3-24 and 92-171R5, and race 4 isolate 90-2, of *Pyrenophora tritici-repentis*. Leaves are shown 168 h after inoculation with the respective isolates. Line 6B662 is sensitive to Ptr ToxB and susceptible to isolates Alg3-24 and 92-171R5. 'Erik' is insensitive to Ptr ToxB and resistant to isolates Alg3-24 and 92-171R5. Both wheat genotypes are resistant to isolate 90-2.

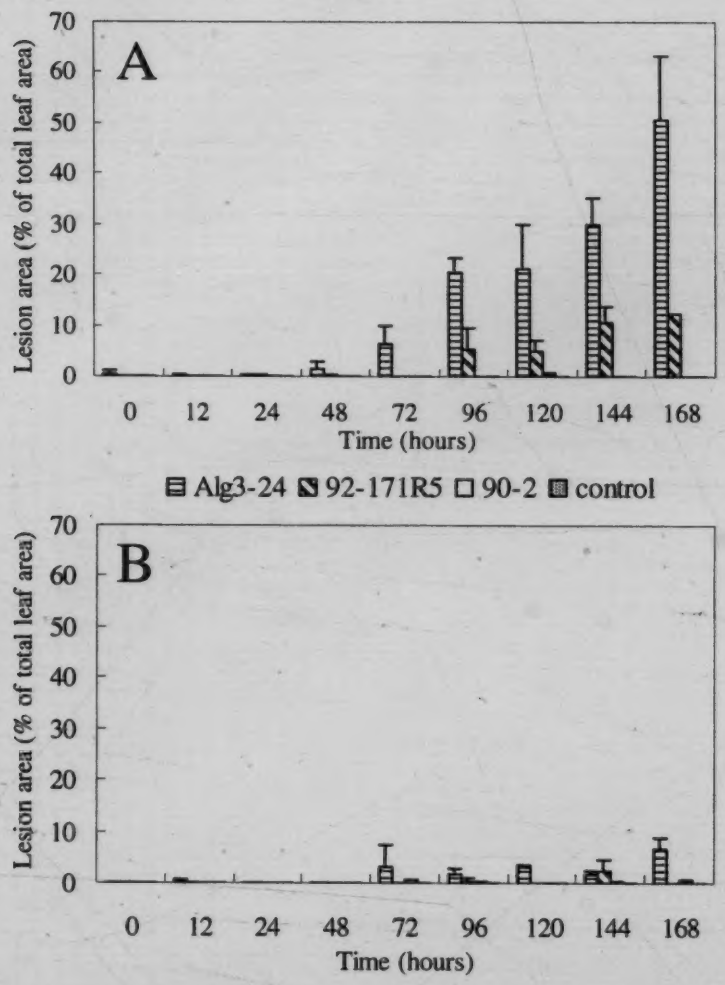

Figure 2-2. Quantification of foliar lesion development on wheat line 6B662 (A) and cultivar Erik (B) in response to inoculation with race 5 isolates Alg3-24 and 92-171R5, and race 4 isolate 90-2, of *Pyrenophora tritici-repentis*. Controls were inoculated with water only.Leaves were analyzed using image analysis software. Lesion area was calculated as a percentage of the total leaf area and the mean from three biological repetitions of the experiment are shown. Error bars indicate the standard deviation.

this isolate at 168 h. Neither isolate produced chlorosis on the Ptr ToxB-insensitive genotype 'Erik,' although small, localized necrotic lesions typical of a resistant reaction (Lamari and Bernier 1989a) were observed (Fig. 2-1). While inoculation with race 4 isolate 90-2 caused the development of a few small chlorotic flecks on line 6B662, this isolate failed to induce any significant symptoms on either genotype, confirming its avirulent designation (Figs. 2-1 and 2-2). No symptoms were observed on control plants inoculated only with water (results not shown). Single spore isolates of *P. tritici-repentis* were successfully re-isolated from leaves of line 6B662 and 'Erik' 168 h after inoculation with Alg3-24, 92-171R5, or 90-2, in all repetitions of the experiment.

2.3.2 Pigment Concentration

Total chlorophyll concentration in the leaves of wheat genotypes 6B662 and 'Erik' was in the 2.0 to 2.5 mg/g tissue range at the time of inoculation with *P. tritici-repentis* (Fig 2-3). In line 6B662, chlorophyll levels began to decrease 48 to 72 h after inoculation with race 5 isolates Alg3-24 and 92-171R5, and continued to decline throughout the time-course of the study (Fig. 2-3A). By 168 h, the level of total chlorophyll had dropped to 0.99 mg/g tissue in response to inoculation with Alg3-24, or 1.78 mg/g tissue in response to inoculation with 92-171R5. Similar rates of decline were observed for chlorophyll *a* and *b*, and the ratio between the two pigments remained constant (results not shown). A decline in total chlorophyll concentration was also observed in 'Erik' in response to inoculation with isolates

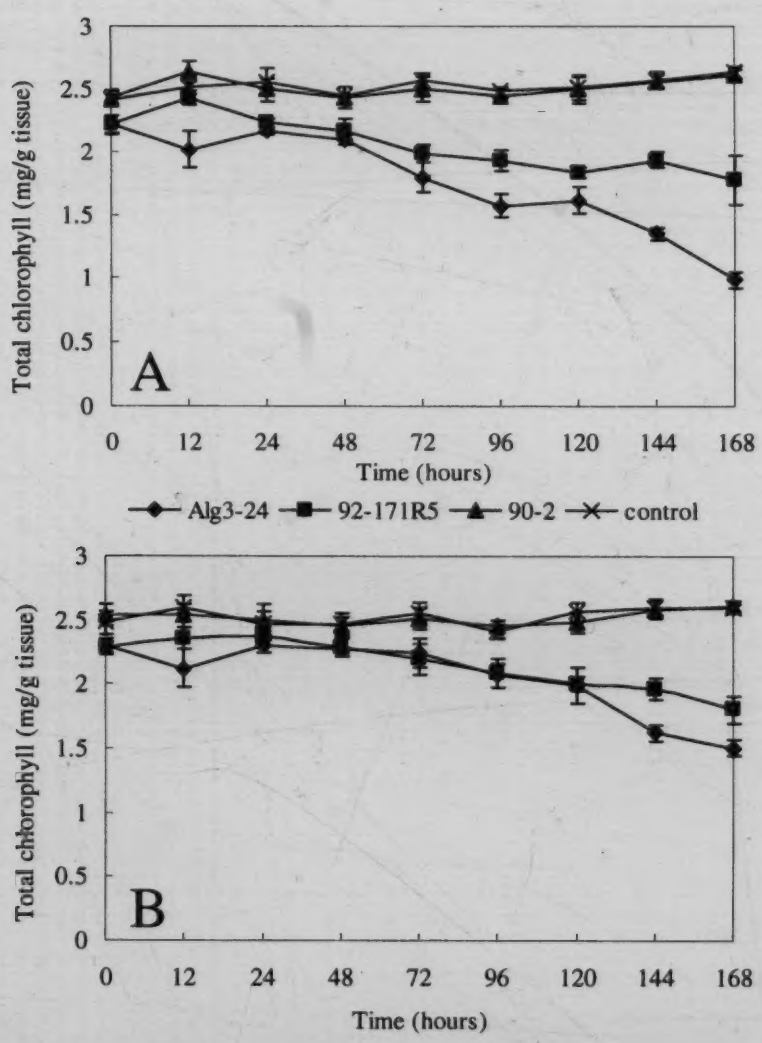

Figure 2-3. Total chlorophyll concentration over time after inoculation of wheat line 6B662 (A) and cultivar Erik (B) with race 5 isolates Alg3-24 and 92-171R5, and race 4 isolate 90-2, of *Pyrenophora tritici-repentis*. Controls were inoculated with water only. The ratio between chlorophyll *a* and *b* remained constant in all treatments and is therefore not shown. Chlorophyll concentration was calculated using the equations of Lichtenthaler (1987), and the means from three biological repetitions of the experiment are shown. Error bars indicate the standard errors.

Alg3-24 and 92-171R5, but it was of a much smaller magnitude (Fig. 2-3B). Total chlorophyll concentration in 'Erik' decreased from 2.3 to 1.5 mg/g tissue at 168 h after inoculation with Alg3-24, while a decrease from 2.3 to 1.8 mg/g tissue was observed in response to 92-171R5 (Fig. 2-3B). Inoculation with race 4 isolate 90-2 did not cause any detectable decline in total chlorophyll concentration in either wheat genotype (Fig. 2-3). Similarly, chlorophyll content in water-inoculated seedlings also remained constant at all time points examined.

The trends in carotenoid concentration were similar to those observed with respect to chlorophyll. Carotenoid concentration was in the 0.60 to 0.70 mg/g tissue in both host genotypes at the time of inoculation, but declined to 0.40 mg/g tissue at 168 h on 6B662 after inoculation with Alg3-24, and to 0.57 mg/g tissue after inoculation with 92-171R5 (Fig. 2-4A). A smaller decrease was observed in 'Erik' in response to inoculation with these two isolates. In this genotype, carotenoid concentration declined from 0.69 to 0.55 mg/g tissue at 168 h after inoculation with Alg3-24, and from 0.66 to 0.50 mg/g tissue after inoculation with 92-171R5 (Fig. 2-4B). Observed declines of both total chlorophyll and carotenoid concentration in 'Erik' could be correlated with increasing necrotic lesions per total amount of leaves. As was observed with respect to chlorophyll, inoculation of either genotype with isolate 90-2 or water had no significant effect on carotenoid concentration.

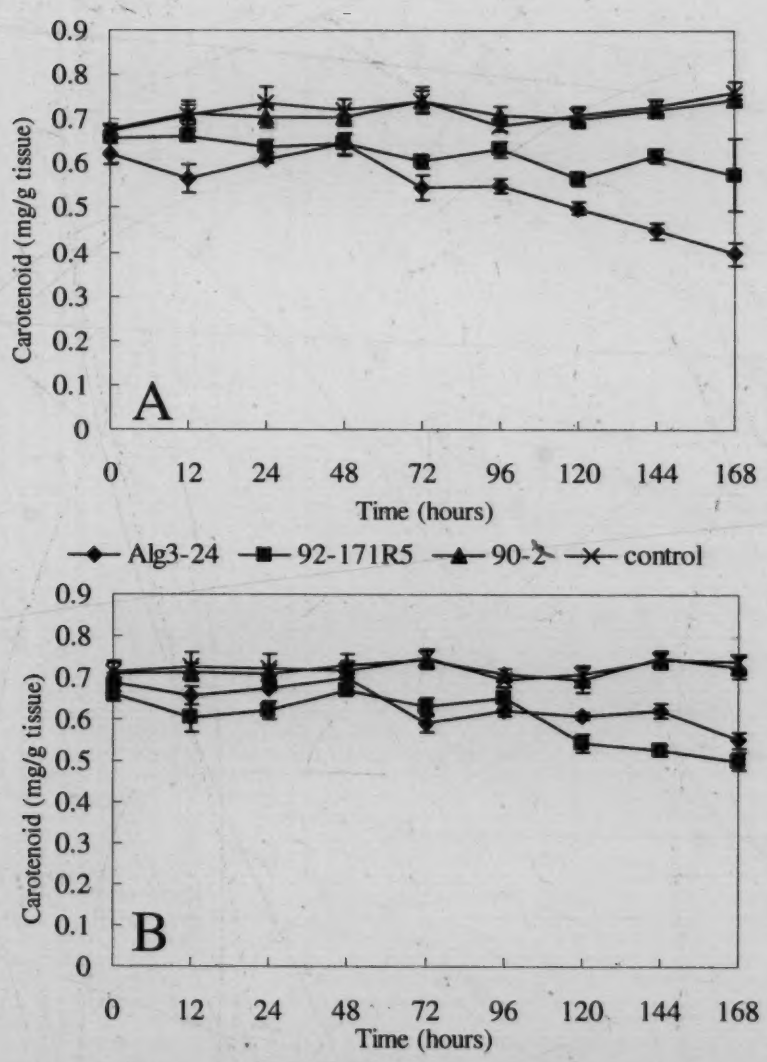

Figure 2-4. Carotenoid concentration over time after inoculation of wheat line 6B662 (A) and cultivar Erik (B) with race 5 isolates Alg3-24 and 92-171R5, and race 4 isolate 90-2, of *Pyrenophora tritici-repentis*. Controls were inoculated with water only. Carotenoid concentration was calculated using the equations of Lichtenthaler (1987), and the means from four biological repetitions of experiment are shown. Error bars indicate the standard errors.

2.3.3 Conventional RT-PCR

Conventional RT-PCR confirmed the presence of a *ToxB* transcript in conidia of race 4 isolate 90-2 and race 5 isolates Alg3-24 and 92-171R5, with a single product of the expected 307 bp (for 90-2) or 304 bp (for Alg3-24 and 92-171R5) size amplified in each case (Fig. 2-5). The presence of *ToxB* mRNA could also be detected in 5, 10, 15 and 20 day-old liquid-grown mycelia of Alg3-24, but only in 20 day-old mycelia of 92-171R5 (Fig. 2-5). No product was observed at any time-point in mycelia of isolate 90-2. In total RNA extracted from wheat leaves inoculated with Alg3-24, a *ToxB* transcript could be detected at all times tested except 0 h, with the intensity of the corresponding band becoming slightly stronger after 12 h (Fig. 2-6). This pattern was observed on both the Ptr ToxB-sensitive line 6B662 and the insensitive cv. Erik. In the case of 92-171R5, a strong band corresponding to *ToxB* was first detectable 24 h after inoculation, but the band intensity became weaker after that, and a only a very faint band was observed from 48 to 168 h (Fig. 2-6). As observed with Alg3-24, the pattern of *ToxB* expression by 92-171R5 was similar in both 6B662 and 'Erik.' In the case of 90-2, no *ToxB* transcript was detectable at any time point on either host genotype (Fig. 2-6). No amplicons were observed in the negative controls.

2.3.4 Actin Gene Sequences

Amplification of genomic DNA from *P. tritici-repentis* isolates Alg3-24, 92-171R5 and 90-2 with the actin-specific primers Act-1 and Act-5ra (Voigt and

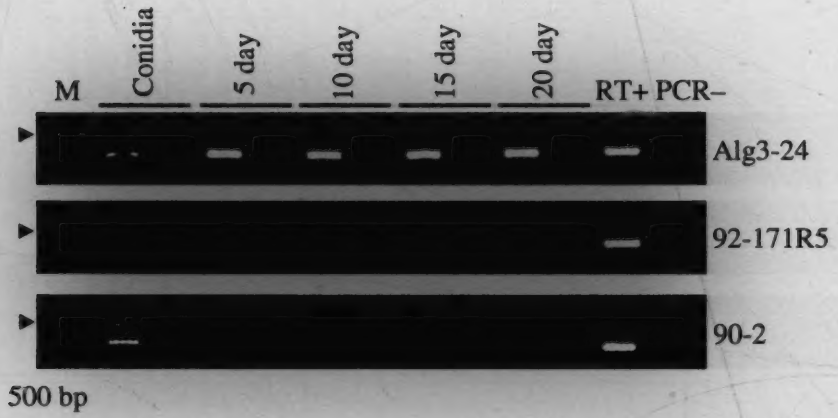

Figure 2-5. Reverse-transcriptase (RT)-PCR analysis of total RNA from conidia and 5, 10, 15 and 20 day-old mycelia of isolates Alg3-24 (top), 92-171R5 (middle) and 90-2 (bottom) of *Pyrenophora tritici-repentis*. Total RNA was reverse-transcribed, amplified with the *ToxB*-specific primers *ToxBF3* and *ToxBR3*, and the amplicons visualized on 1.2% agarose gels. There are two lanes per treatment: (1) one contains the sample obtained when RT-PCR was conducted with all required components, and the other (2) is a control that was not treated with RT prior to PCR (to ensure the absence of genomic DNA). In addition, a positive control (RNA extracted from mycelia of 20 day-old cultures of Alg3-24, RT+) and a negative control (all reaction components except a DNA template, PCR-) were included in the assays to ensure proper reaction conditions and the absence of DNA contaminants in the reaction mixtures. RT-PCR analysis was repeated 3 times for each of the 3 repetitions of the experiment, and results from a typical run are shown.

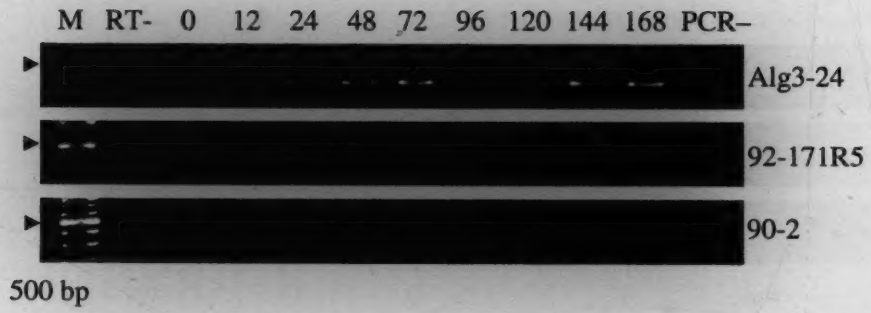

Figure 2-6. Reverse-transcriptase (RT)-PCR analysis of total RNA extracted from leaf tissue of wheat line 6B662 after inoculation with isolates Alg3-24 (top), 92-171R5 (middle) and 90-2 (bottom) of *Pyrenophora tritici-repentis*. Total RNA was reverse-transcribed, amplified with the *ToxB*-specific primers *ToxBF3* and *ToxBR3*, and the amplicons visualized on 1.2% agarose gels. Very similar *ToxB* expression patterns were obtained on wheat cultivar Erik and are not shown. Leaf tissue was sampled at 0, 12, 24, 48, 72, 96, 120, 144 and 168 h after inoculation with each isolate. Negative controls to which no RT was added (RT-) or which included all reaction components except a DNA template (PCR-) were also included. RT-PCR analysis was repeated 3 times for each of the 3 repetitions of the experiment, and results from a typical run are shown.

Wostemeyer 2000; Voigt et al. 2005) yielded a single PCR product approximately 900 bp in size for each isolate. Nucleotide sequences of 893, 726, and 872 bp in length were obtained for the amplicons from Alg3-24 (GenBank accession no. EF180087), 92-171R5 (accession no. EF180088), and 90-2 (accession no. EF180089), respectively (see Appendix). Sequence analysis revealed that the gene shared 99% homology over 721 bp amongst the three isolates. In contrast, comparison with an actin gene sequence from wheat available in GenBank (accession no. AY423548) revealed only 72 % similarity over 545 bp.

2.3.5 qRT-PCR

Analysis of *ToxB* expression by qRT-PCR allowed comparison of relative transcript levels among the three *P. tritici-repentis* isolates included in this study. This analysis revealed that while expression of *ToxB* was greatest in 5 to 20 day-old mycelia of Alg3-24, a very low level of transcript was also detectable in mycelia of isolates 90-2 and 92-171R5 (Table 2-1). The amount of transcript increased in all isolates during the time course of the study, but the level in Alg3-24 was nearly 20,000-fold greater than in 92-171R5 and 25,000-fold greater than in 90-2 by 20 days. The qRT-PCR analysis also confirmed that the amount of *ToxB* transcript was greater in conidia of isolates 90-2 and 92-171R5 than in liquid-grown mycelia, at all time-points tested. In contrast, *ToxB* expression in Alg3-24 was greater in mycelia than conidia, particularly after day 5. Nevertheless, the abundance of the transcript was 2 to 3 orders of magnitude greater in conidia of Alg3-24 than in conidia of 90-2 or 92-171R5.

Table 2-1. Quantitative real-time PCR (qRT-PCR) analysis of the relative expression of the *ToxB* gene in conidia and mycelia of *Pyrenophora tritici-repentis* isolates Alg3-24, 92-171R5 and 90-2

	Relative Expression[†]		
Isolate	Alg3-24	92-171R5	90-2
5 days	5.00×10^{4}	1.87	1.00
	(4.63×10^{4}-5.39×10^{4})	(1.51-2.29)	(0.74-1.34)
10 days	4.76×10^{5}	6.36	1.59×10^{1}
	(3.56×10^{5}-6.35×10^{5})	(5.28-7.67)	(1.29×10^{1}-1.94×10^{1})
15 days	8.34×10^{5}	3.5×10^{1}	1.62×10^{1}
	(6.37×10^{5}-1.09×10^{6})	(2.22×10^{1}-5.53×10^{1})	(8.51-3.09×10^{1})
20 days	1.56×10^{6}	7.82×10^{1}	6.31×10^{1}
	(1.37×10^{6}-1.76×10^{6})	(7.35×10^{1}-8.32×10^{1})	(5.49×10^{1}-7.25×10^{1})
conidia	1.90×10^{4}	3.68×10^{1}	2.44×10^{2}
	(1.49×10^{4}-2.41×10^{4})	(2.79×10^{1}-4.85×10^{1})	(2.08×10^{2}-2.86×10^{2})

[†]*ToxB* expression data was normalized using an actin endogenous control; relative expression was calibrated against *ToxB* transcript levels in 5 day-old mycelia of isolate 90-2 according to the comparative C_T method for relative quantification of gene expression (Livak and Schmittgen 2001).

Table 2-2. Quantitative real-time PCR (qRT-PCR) analysis of the relative expression of the *ToxB* gene by isolates Alg3-24, 92-171R5 and 90-2 of *Pyrenophora tritici-repentis*, at various times after inoculation onto wheat line 6B662 and cultivar Erik

	Relative Expression[†]					
	6B662			Erik		
	Alg3-24	92-171R5	90-2	Alg3-24	92-171R5	90-2
0 h	5.24 (4.25-6.45)	3.71 (2.46-5.57)	1.00 (0.81-1.23)	1.33 (0.85-2.07)	0.27 (0.2-0.36)	1.00 (0.62-1.61)
12 h	1.18×10^{3} (7.34×10^{3}-1.88×10^{4})	5.02×10^{1} (3.42×10^{1}-7.35×10^{1})	0.19 (0.15-0.23)	1.35×10^{3} (1.07×10^{3}-1.69×10^{3})	1.30×10^{1} (9.25-1.82×10^{1})	0.12 (0.11-0.14)
24 h	1.31×10^{4} (1.99×10^{4}-8.66×10^{3})	1.36×10^{2} (1.15×10^{2}-1.60×10^{2})	0.10 (0.08-0.11)	4.43×10^{3} (3.01×10^{3}-6.03×10^{3})	3.87×10^{1} (2.86×10^{1}-4.98×10^{1})	0.21 (0.11-0.55)
48 h	5.6×10^{3} (5.07×10^{3}-6.08×10^{3})	9.19 (5.98-1.41×10^{1})	0.42 (0.34-0.52)	5.08×10^{3} (4.12×10^{3}-6.25×10^{3})	7.46 (5.06-1.10×10^{1})	0.27 (0.13-0.52)
72 h	8.6×10^{3} (2.49×10^{3}-1.01×10^{4})	2.16 (1.58-2.94)	1.13 (0.86-1.33)	4.88×10^{3} (4.09×10^{3}-5.79×10^{3})	1.52 (1.23-1.89)	0.44 (0.37-0.52)
96 h	3.04×10^{3} (3.72×10^{3}-2.48×10^{3})	0.42 (0.25-0.72)	8.88 (5.73-1.28×10^{1})	2.50×10^{3} (1.91×10^{3}-3.28×10^{3})	1.07 (0.83-1.37)	1.73 (1.10-2.69)
120 h	3.33×10^{3} (2.80×10^{3}-3.95×10^{3})	0.33 (0.19-0.57)	1.05 (0.82-1.33)	5.79×10^{3} (5.04×10^{3}-6.65×10^{3})	0.41 (0.27-0.62)	0.70 (0.45-1.07)
144 h	1.63×10^{3} (1.34×10^{3}-1.98×10^{3})	0.09 (0.06-0.12)	1.87 (1.01-3.43)	3.57×10^{3} (3.15×10^{3}-4.03×10^{3})	0.07 (0.04-0.12)	1.34 (0.94-1.89)
168 h	1.39×10^{3} (1.1×10^{3}-1.75×10^{3})	0.06 (0.04-0.08)	1.2×10^{1} (5.66-2.56×10^{1})	6.13×10^{2} (5.26×10^{2}-7.14×10^{2})	0.04 (0.02-0.06)	2.17 (0.88-5.35)

[†]*ToxB* expression data was normalized using an actin endogenous control; relative expression was calibrated against *ToxB* transcript levels at 0 h for isolate 90-2 on 6B662 or 'Erik' according to the comparative C_T method for relative quantification of gene expression (Livak and Schmittgen 2001).

The *ToxB* expression pattern was also quantified in planta after inoculation of the Ptr ToxB-sensitive and insensitive wheat genotypes 6B662 and 'Erik' with isolates Alg3-24, 92-171R5 and 90-2. Abundance of the *ToxB* transcript was much higher in leaves inoculated with Alg3-24 than in leaves inoculated with 92-171R5 or 90-2 (Table 2-2). Transcript abundance was also higher in leaves inoculated with 92-171R5 than with 90-2, at least from 12 to 48 h after inoculation. Interestingly, the relative pattern of *ToxB* expression by each isolate was similar in both hosts (Table 2-2). In the case of the race 5 isolates Alg3-24 and 92-171R5, relative transcript abundance increased rapidly after inoculation, peaking at 24 h (Table 2-2). In 6B662 leaves inoculated with Alg3-24, relative *ToxB* transcript abundance increased 2498-fold from 0 to 24 h, while in 'Erik,' it increased 3262-fold over the same period. Similarly, after inoculation with 92-171R5, abundance of the *ToxB* transcript increased 37 and 140-fold in 6B662 and 'Erik,' respectively, from 0 to 24 h. However, after 24 h, relative expression of the *ToxB* gene by isolates Alg3-24 and 92-171R5 slowly declined in both hosts, although this decline was strongest in 92-171R5 (Table 2-2). By 168 h after inoculation, *ToxB* transcript abundance in 92-171R5-inoculated tissue was only a fraction of that at 0 h, while in Alg3-24-inoculated tissue, it was still several hundred-fold greater. The expression pattern for the *ToxB* gene in race 4 isolate 90-2 was markedly different (Table 2-2). Unlike in tissues inoculated with isolates Alg3-24 and 92-171R5, there was no large increase in the level of *ToxB* transcript after inoculation with 90-2, with relative abundance remaining very low throughout the time-course of the study (Table 2-2). Indeed, with 90-2, the greatest

increase in *ToxB* transcript level was not observed until 168 h after inoculation in line 6B662, when it was only 12-fold greater than at time 0 h. On 'Erik,' the highest abundance was also observed at 168 h, but the increase was only 2.2-fold relative to 0 h. No amplification signals were detected in the negative controls.

2.4. Discussion

The host-specific toxins produced by *Pyrenophora tritici-repentis* serve as pathogenicity factors for the fungus and therefore play an important role in the development of tan spot of wheat (Strelkov and Lamari 2003). Two of these toxins, Ptr ToxA and Ptr ToxB, have been shown to be proteins, and are encoded by the *ToxA* and *ToxB* genes, respectively (Ballance et al. 1996; Ciuffetii et al. 1997; Martinez et al. 2001; Strelkov and Lamari 2003). However, while the *ToxA* gene is found only in isolates of *P. tritici-repentis* that possess Ptr ToxA activity, homologs of *ToxB* have also been reported from isolates that do not possess, or show reduced, Ptr ToxB activity (Strelkov and Lamari 2003; Martinez et al. 2004; Strelkov et al. 2006). In the present study, we used qRT-PCR with TaqMan probes to compare expression of the *ToxB* gene in virulent, low virulence and avirulent isolates of the fungus, in order to gain insights into the role of Ptr ToxB as a pathogenicity factor. Quantitative RT-PCR is a sensitive and reliable method for quantifying gene expression, which allows detection of amplification products in real-time, as opposed to relying on end-point measurements of accumulated products (Schena et al. 2004).

Our results confirmed that the *ToxB* gene is transcribed, albeit at very different levels, in isolates Alg3-24, 92-171R5 and 90-2 of *P. tritici-repentis*. In mycelia grown in liquid culture, relative transcript abundance was greatest in Alg3-24 at all time-points examined (Table 2-1). Levels were many folds lower in 92-171R5 and 90-2, and transcript abundance in mycelia of these two isolates was within the same order of magnitude. This very low abundance in mycelia probably explains our results with conventional RT-PCR, in which no amplicons could be observed in cultures of 90-2 at any time, or in cultures of 92-171R5 at most time-points examined (Fig. 2-5). It may also explain previous reports suggesting that *ToxB* was not expressed in mycelia of race 4 isolates (Martinez et al. 2004; Strelkov et al. 2006), or in mycelia of the low-virulence race 5 isolate 92-171R5 (Strelkov et al. 2006). In those reports, gene expression was examined by conventional RT-PCR and Northern blotting, which are less sensitive techniques.

Although analysis of expression in culture was useful to confirm transcription of *ToxB* and its related forms, the expression pattern of the gene in conidia and in toxin-sensitive and insensitive host tissue may be more relevant with respect to disease development. A number of fungal species, such as *Botryodiplodia theobromae* (Knight and Van Etten 1976), *Mucor racemosus* (Linz and Orlowski 1982) and *Neurospora tetrasperma* (Plesofsky-Vig et al. 1992), are known to produce stored mRNA that is translated upon germination. Given the importance of Ptr ToxB in establishing a compatible interaction between host and pathogen, detecting its transcript in conidia was not surprising, as its presence would

presumably enable faster toxin production during the early stages of infection. As expected, the greatest amount of transcript was found in conidia of the most virulent isolate, Alg3-24 (Table 2-1). However, approximately 6.6× more transcript was detected in conidia of 90-2 than 92-171R5, which is counter-intuitive, since the former is avirulent and the latter is low virulence. Nevertheless, analysis of the relative abundance of the *ToxB* transcript in planta confirmed that expression of the gene was greater in race 5 isolates Alg3-24 and 92-171R5 than in race 4 isolate 90-2 (Table 2-2). Furthermore, while the level of expression was highest in Alg3-24, the general expression pattern of the *ToxB* gene was quite similar in Alg3-24 and 92-171R5, but differed from isolate 90-2 (Table 2-2).

In the race 5 isolates, the transcript was most abundant at 24 h after inoculation, preceding the development of chlorosis by approximately 48 h (Fig. 2-2) in the toxin-sensitive genotype 6B662. Unfortunately, high titer antibodies were not available to correlate gene expression with protein levels, and we could not assess whether the quantity of Ptr ToxB in infected tissue also peaked at 24 h. However, the delayed effect of the toxin in inducing chlorosis (Strelkov et al. 1998) could explain why the development of this symptom occurred after transcript levels had reached their maximum. In race 4 isolate 90-2, relative expression of *ToxB* remained very low throughout the time course, with no peak observed at 24 h and maximum transcript abundance detected at 168 h (Table 2-2). This low level of expression may contribute to the development of only trace levels of chlorosis in response to 90-2 (Fig. 2-1). However, the form of *ToxB* in race 4 isolates exhibits only 86% similarity over the

ORF to 'wild-type' *ToxB* from race 5 isolates (Strelkov and Lamari 2003; Martinez et al. 2004), and the toxin protein it encodes appears to be much less active (Kim and Strelkov 2006). Therefore, the main basis for the observed lack of Ptr ToxB activity may also be related to the form of the protein encoded, combined with a much lower level of expression.

The differences observed in expression level and pattern, particularly between the race 4 and race 5 isolates, most likely reflect the significant differences that have been reported upstream of the ORF in these isolates (Martinez et al. 2004; Strelkov et al. 2006). However, in addition to the differences in regulation of expression, the differential expression of this gene may also be at least partially attributable to variation in *ToxB* copy number. In the virulent isolate Alg3-24, the *ToxB* gene is found as 8-10 copies, while in 92-171R5, only two copies are found, and in 90-2, the *ToxB* homolog exists as a single copy (Strelkov et al. 2006).

The *ToxB* expression profiles in all three isolates were similar in both the Ptr ToxB-sensitive and insensitive hosts (Table 2-2). Furthermore, the isolates, including avirulent isolate 90-2, could be recovered from inoculated leaves of both genotypes 7 days after inoculation. Previous research has demonstrated that *P. tritici-repentis* can germinate, form appressoria, penetrate epidermal cells and grow into the intercellular space of the mesophyll in both resistant and susceptible wheat genotypes (Larez et al. 1986; Lamari and Bernier 1989b). Larez et al. (1986) postulated that, because they observed no obvious histological or cytological structural mechanisms for restricting the pathogen, a molecular mechanism for resistance was at play. Since then, the *P.*

tritici-repentis/wheat interaction has been shown to follow the toxin model, in which recognition between a host and pathogen leads to compatibility (susceptibility), as opposed to incompatibility (resistance) in the classical gene-for-gene model (Strelkov and Lamari 2003). Therefore, the molecular mechanism involved in this pathosystem confers susceptibility, rather than resistance, in the absence of structural mechanisms for restricting pathogen development.

In the present study, all three isolates tested became established in the host tissue, and (at least in the case of the race 5 isolates) expressed the *ToxB* gene, leading to the production of Ptr ToxB and the development of chlorosis on line 6B662. Although the *ToxB* expression profile was similar in 'Erik,' the insensitivity of this genotype ensured that toxin-induced chlorosis did not develop. Hence, lesion development was greatly restricted in this cultivar (Fig. 2-2B). Therefore, the ability of the fungus to produce (active) Ptr ToxB was crucial for the progress of the disease. Nevertheless, microscopic analysis suggested that the rate at which infective structures (i.e. appressoria) are formed by the virulent, low virulence and avirulent isolates varies, suggesting that other factors may also contribute to the general pathogenic ability of *P. tritici-repentis* (Chapter 3). Regardless of whatever other features may be involved, it is clear that the production of Ptr ToxB (or one of the other Ptr toxins) remains one of the most important in establishing the tan spot disease.

2.5 Literature Cited

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3.0 Microscopic Analysis of the Interaction between Wheat and Ptr ToxB-Producing Isolates of *Pyrenophora tritici-repentis*

3.1 Introduction

Tan spot, caused by the homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.), is an important foliar disease of wheat (*Triticum aestivum L.*). The fungus has a wide host range and can infect at least 33 species in the Gramineae (Krupinsky 1982; 1992; Hosford 1982). On susceptible wheat genotypes, *P. tritici-repentis* induces two distinct symptoms: tan necrosis and/or extensive chlorosis. Fungal isolates were initially classified into four pathotypes (P1: $nec^{+}chl^{+}$, P2: $nec^{+}chl^{-}$, P3: $nec^{-}chl^{+}$, and P4: $nec^{-}chl^{-}$) based on their ability to induce these symptoms on specific wheat differentials (Lamari and Bernier 1989a). However, pathotype classifications could not accommodate situations in which the same symptom was induced on different host genotypes. As a consequence, a race classification system, based on the reaction of isolates on individual wheat genotypes, was later adopted (Lamari et al. 1995). This system can accommodate any number of races and is limited only by the uniqueness and number of the differential wheat genotypes used (Lamari et al. 1995).

The ability of isolates of *P. tritici-repentis* to infect and cause symptoms on specific wheat genotypes is linked to their ability to produce one or more of the host-specific Ptr toxins associated with the pathogen (Strelkov and Lamari 2003). These toxins, at least three of which are known, appear to be pathogenicity factors and are essential for disease development. Ptr ToxA (syn. Ptr toxin, Ptr necrosis toxin,

and ToxA [Ciuffetti et al. 1998]) is encoded by a single copy gene, termed *ToxA* (Ciuffetti et al. 1998), and is a 13.2 kDa protein that induces necrosis in sensitive wheat genotypes (Ballance et al. 1989; Tomás et al. 1990; Tuori et al. 1995; Zhang et al. 1997). Ptr ToxB (syn. Ptr chlorosis toxin [Ciuffetti et al. 1998]) induces chlorosis in sensitive wheat lines/cultivars, and is also a protein with a 6.61 kDa mass (Strelkov et al. 1999). The Ptr ToxB protein is encoded by the *ToxB* gene, which occurs in 8-10 copies in the virulent race 5 isolates of the fungus from which it was originally cloned (Strelkov 2002; Martinez et al. 2004; Strelkov et al. 2006). Ptr ToxC, which has been only partially characterized, appears to be a polar, low molecular mass molecule that also induces chlorosis, but not on the same wheat genotypes as Ptr ToxB (Effertz et al. 2002). There are preliminary reports of the production of at least two other host-specific toxins by the pathogen (Ali et al. 2002; Manning et al. 2002).

In addition to being found in isolates of *P. tritici-repentis* that possess strong Ptr ToxB activity, homologs of the *ToxB* gene have been identified in isolates that possess little or no toxin activity. In the avirulent race 4 isolate 90-2, the *ToxB* homolog is found as a single copy that shares 86% similarity to the wild-type gene over the open reading frame (ORF) (Strelkov 2002; Strelkov and Lamari 2003). A form of the *ToxB* gene has also been identified in low-virulence race 5 isolate 92-171R5. In the case of 92-171R5, the *ToxB* gene is identical to the wild-type over the region of the ORF encoding the mature toxin, but appears to exhibit substantial sequence differences in the upstream flanking regions (Strelkov et al. 2006). Moreover, the form of *ToxB* in

92-171R5 is found as only two copies, as opposed to 8-10 copies in fully virulent race 5 isolates (Martinez et al. 2004; Strelkov et al. 2006).

Previous research using quantitative real-time (qRT)-PCR (Chapter 2) revealed significant differences in *ToxB* gene expression among isolates Alg3-24, 92-171R5 and 90-2 of *P. tritici-repentis*. The expression of *ToxB* was generally correlated with the pathogenic ability of the isolates. Therefore, gene expression was highest in conidia and mycelia of the virulent isolate Alg3-24, although *ToxB* transcript was also detected in conidia and mycelia of the low virulence isolate 92-171R5 and the avirulent isolate 90-2, confirming that the *ToxB* homologs in the latter two isolates are transcribed. Similarly, *ToxB* transcript abundance in host leaf tissue was highest after inoculation with the virulent isolate, followed by the low-virulence isolate, and lowest after inoculation with the avirulent isolate, with the overall pattern of gene expression similar in the two race 5 isolates (virulent and low virulence), but very different in the race 4 isolate (avirulent) (Chapter 2). Expression of the gene was similar in Ptr ToxB-sensitive and insensitive wheat genotypes.

The detection of *ToxB* transcript in toxin-sensitive and insensitive leaf tissue, after inoculation with virulent, low-virulence and avirulent isolates, indicates that even in incompatible interactions, *P. tritici-repentis* can establish itself in the host (Chapter 2). This conclusion is further strengthened by the fact that the fungus could be re-isolated from resistant leaves 1 week after inoculation (Chapter 2), and confirms the previous suggestion that the host specificity and virulence conferred by the Ptr toxins may be superimposed on the general pathogenic ability of the fungus (Lamari

et al. 1998). Even in incompatible interactions, conidia germinate, form appressoria, penetrate epidermal cells and grow into the intercellular space of the mesophyll, where further growth is severely restricted (Larez et al. 1986; Lamari and Bernier 1989a). In addition, acquisition of toxin-producing ability (Ptr ToxA), after transformation of an avirulent isolate with the *ToxA* gene, was shown to be a sufficient condition for virulence on a Ptr ToxA-sensitive wheat genotype (Ciuffetti et al. 1997).

However, previous histological and cytological studies have focused almost exclusively on necrosis-inducing (Ptr ToxA-producing) isolates of *P. tritici-repentis*. To our knowledge, only one study (Lamari and Bernier 1989a) examined the cytology of an incompatible interaction involving an isolate that could also induce chlorosis, but this was race 3-type or Ptr ToxC-induced chlorosis. Furthermore, previous reports have described the incompatible interaction that occurs between virulent (toxin-producing) isolates and resistant or toxin-insensitive wheat genotypes, rather than between avirulent (toxin non-producing) isolates and susceptible or toxin-sensitive genotypes. Therefore, the present study was aimed at characterizing the cytology and histopathology of the interaction between Ptr ToxB-sensitive and insensitive wheat genotypes and fungal isolates differing in virulence and Ptr ToxB-producing ability. The information obtained may be helpful in further elucidating the role of Ptr ToxB in tan spot disease development and in understanding the nature of quantitative variation in the virulence of *P. tritici-repentis*.

3.2 Materials and Methods

3.2.1 Terminology

The terms 'susceptibility' and 'resistance' are used to describe the host reaction to *P. tritici-repentis*, while 'sensitivity' and 'insensitivity' refer to the host reaction to the fungal toxins.

3.2.2 Fungal Isolates

Three isolates of *P. tritici-repentis*, representing races 4 and 5 of the pathogen, were used in this study. Race 4 isolate 90-2, which lacks Ptr ToxB activity, was obtained from the Canadian prairies and is avirulent (Lamari et al. 1998). Race 5 isolates Alg3-24 and 92-171R5 were collected in eastern Algeria and the Saskatchewan-Manitoba border (Lamari et al. 1995, Strelkov et al. 2002), respectively, and while both possess Ptr ToxB activity, that activity is much weaker in 92-171R5 (Strelkov et al. 2002). Isolates were grown in Petri dishes on V8-potato dextrose agar (PDA) and conidial inoculum was produced as previously described (Lamari and Bernier 1989b).

3.2.3 Plant Material and Inoculation

Two hexaploid wheat genotypes, 6B662 and 'Erik,' were used in this study. Line 6B662 is Ptr ToxB-sensitive, resistant to race 4 and susceptible to race 5, while 'Erik' is Ptr ToxB-insensitive and resistant to both races. Five to six evenly spaced seeds were sown in 10 cm plastic pots filled with Metro-Mix 220 soil (W.R.Grace and Co.,

Ajax, ON), and seedlings were maintained in a greenhouse at 20 °C/18 °C (day/night) with a 16 h photoperiod at 250 μmol $m^{-2}s^{-1}$ (natural light supplemented with artificial lighting) until inoculation at the 2-3 leaf stage (14 days after seedling). The seedlings were inoculated with a suspension of 3500 conidia per ml to which 10 drops of Tween 20 (polyoxyethylene sorbitol monolaurate) per liter were added, using a sprayer connected to an air line. Leaves were sprayed until run-off. Negative controls were inoculated with sterile distilled H_2O containing the same concentration of Tween 20. Immediately following inoculation, the plants were placed under darkness in a misting chamber (relative humidity ≥ 95%) for a 24 h period, with continuous leaf wetness provided by an ultrasonic humidifier. After incubation under high humidity, the plants were transferred to a growth chamber and kept at 20 °C/18 °C (day/night) with a 16 h photoperiod (180 μmol $m^{-2}s^{-1}$) at 60 % relative humidity. Plants were watered and fertilized as required.

3.2.4 Conidial Germination

Germination of conidia was examined in vitro using a modification of the method of Imaizumi et al. (1999). Briefly, a 500 μl aliquot of a conidial suspension (3500 conidia per ml) was spread on 2 % (w/v) water agar in a Petri dish and incubated at 25 °C in darkness. After 3, 6, 9, 12 and 24 h, total 60 randomly chosen conidia were observed by bright-field microscopy for percentage germination, number of germ tubes per conidium, and germ tube length. Conidial germination was also examined in planta as described below.

3.2.5 Leaf Tissue Sampling, Preparation and Staining

For cytological observations, tissue from the middle of the second leaf of individual wheat plants was sampled for analysis at 3, 6, 12, 24, 48 and 72 h after inoculation. Leaf segments (1-2 cm in length) were stored in alcoholic lactophenol (3 parts 95 % ethanol, 1 part lactophenol) until examined (Larez et al. 1986; Lamari and Bernier 1989a; Tuite 1969). Samples were cleared by boiling in the alcoholic lactophenol for 3 min, and then stained in lactophenol-cotton blue for 30 min to 1 h, or in 0.2 % Fluorescent Brightener 28 (Sigma, St. Louis, MO) in 0.1 M Tris-HCl buffer (pH 8.5) for 10 min (Dushnicky et al. 1996; Rohringer et al. 1977). After staining, the leaf segments were washed with distilled water and mounted in 100 % glycerol for observation. A total of thirty randomly selected conidia per sample were examined for percentage germination, number of germ tubes, number of appressoria, and papilla formation underneath the appressoria.

Histological studies of infected tissues were conducted for samples collected 120 h after inoculation with the various isolates of *P. tritici-repentis*. Leaves were sampled as above and fixed in formalin-acetic acid-alcohol at 15 °C with pressure for at least one week (O'Brien and McCully 1981). The samples were then fixed with ethanol and toluene and embedded in paraffin. Cross and longitudinal sections of 10 µm thickness were made with a microtome, dehydrated with albumen glycerol, and incubated overnight at 37 °C. Leaf sections were made from the centre of the chlorotic and necrotic flecks. De-waxed sections were dehydrated using absolute toluene and a graded ethanol series (100 %, 90 %, 70 %, and 50 %). The leaf sections

were stained with 0.05 % of toluidine blue O for 1 min, 0.1 % acid fuchsin for 5 min, or 0.2 % Fluorescent Brightener 28 in 0.2 M Tris-HCl buffer, pH 8.5, for 10 min (Dushnicky et al. 1996; Rohringer et al. 1977). After staining, the samples were washed with distilled water and mounted in an aqueous mounting medium (Electron Microscopy Sciences, Washington, PA).

3.2.6 Statistical Analysis

Analyses of variance for multiple comparisons (Tukey's studentized range test) was conducted using SAS 9.1 software (SAS Institute Inc. 2004). As the variances for the germ tube lengths measured in vitro (Table 3-1) were skewed, only the means are presented for this feature.

3.2.7 Microscopic Examination

Material was examined with a Leitz Wetziar Dialux 20 bright-field microscope and a Leica DM-RXA fluorescent microscope using blue light excitation. Brightfield and fluorescence images were recorded with a CoolSNAP cf Digital Camera (Photometrics, Tucson, AZ), or a MacroFire LM CCD Digital Camera (Optronics, Goleta, CA), respectively. Samples were also observed with a Leica SP2 multiphoton confocal microscope and imaging system, with excitation at 421 nm and emission at 450 nm for blue light, 488 nm and 535 nm for green light, and 543 and 560 nm for red light.

3.3 Results

3.3.1 Disease Development

The Ptr ToxB-producing race 5 isolates Alg3-24 and 92-171R5 caused typical chlorosis symptoms on the toxin-sensitive wheat line 6B662. Neither isolate was able to induce chlorosis on the toxin-insensitive cultivar Erik, although small, necrotic lesions typical of a resistant reaction (Lamari and Bernier 1989a) were observed (Fig. 3-1). Symptom development on 6B662 was greater after inoculation with Alg3-24 than with 92-17R5, which is consistent with the virulent and low virulence designations, respectively, of these isolates (Strelkov et al. 2002). The avirulent race 4 isolate 90-2 failed to cause significant symptoms, aside from a few small chlorotic flecks on 6B662, on either wheat genotype (Fig. 3-1).

3.3.2 Conidial Germination on Water Agar

Examination of germinating conidia on 2% (w/v) water agar revealed no significant differences between the three fungal isolates in terms of the percentage germination. After 3 h of incubation, 98.3 to 100% of conidia from each isolate had germinated, producing an average of 1.83 (90-2) to 2.44 (92-171R5) germ tubes per spore. By 6 h, the number of germ tubes per conidium was not significantly different in any isolate. The germ tubes continued to extend for the entire24 h incubation period. The longest germ tubes were produced by the avirulent isolate 90-2, with an average length of 1503 μm at 24 h. Germ tube length for isolates Alg3-24 and 92-171R5 averaged 1108 and 984 μm, respectively, at that time (Table 3-1).

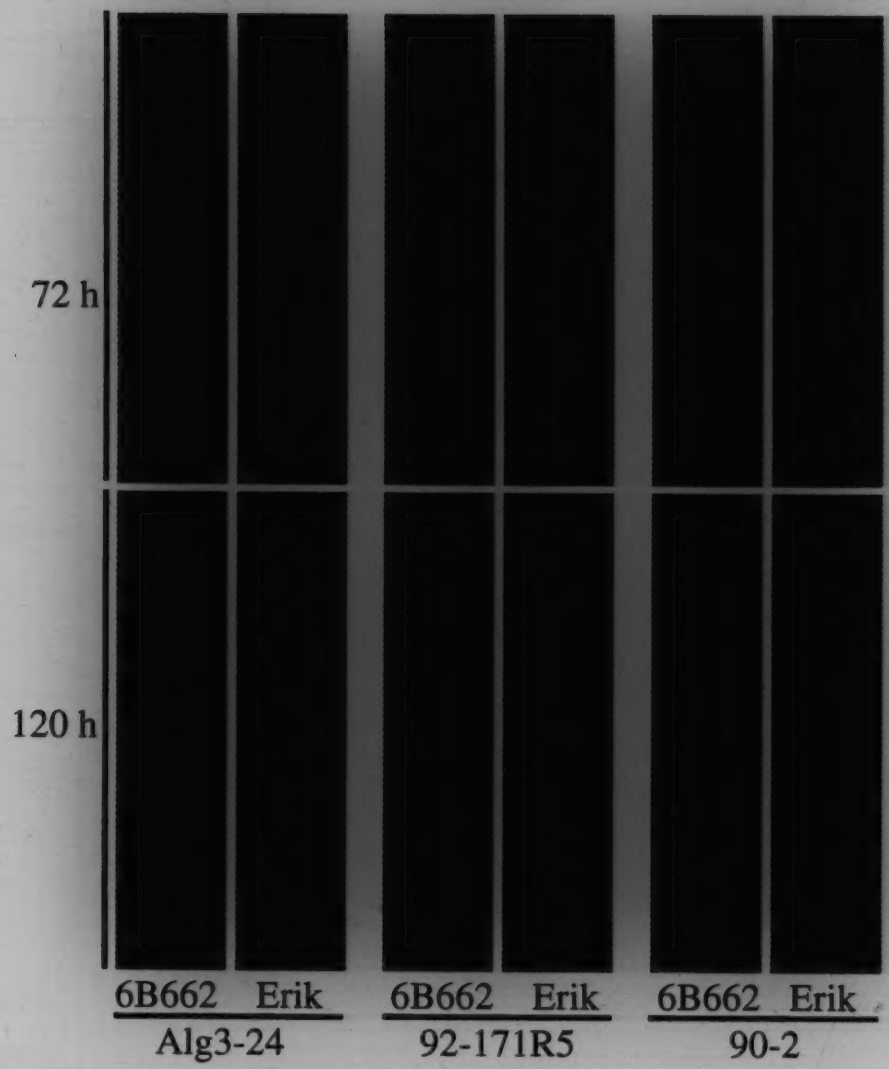


Figure 3-1. Macroscopic symptoms induced on wheat line 6B662 and cultivar 'Erik' 72 and 120 h after inoculation with isolates Alg3-24, 92-171R5 and 90-2 of *Pyrenophora tritici-repentis.*

Table 3-1. Mean number of germ tubes per conidium and average germ tube length in isolates Alg3-24, 92-171R5 and 90-2 of *Pyrenophora tritici-repentis*.[†]

		Length of incubation (h)				
Isolates		3	6	9	12	24
Alg3-24	number‡	2.17 ab	2.52 a	2.35 a	2.65 a	2.67a
92-171R5	number	2.44 a	2.52 a	2.82 a	3.27 a	3.49 a
90-2	number	1.83 b	2.35 a	2.47 a	2.72 a	2.95 a

		Length of incubation (h)				
Isolates		3	6	9	12	24
Alg3-24	length(nm)*	177.39	471.07	557.74	775.65	1108.38
92-171R5	length(nm)	53.07	220.2	341.55	484.58	983.57
90-2	length(nm)	98.92	288.83	534.25	693.12	1502.66

[†]Incubated on 2% (w/v) water agar.

‡Assessed in a total of 60 randomly selected conidia; means followed by a common letter in the same column are not significantly different ($P < 0.001$) as determined by Tukey's Studentized Range (HSD) test.

*As the variances for germ tube length were skewed, only the means are presented for this feature.

3.3.3 Fungal Development in Compatible and Incompatible Interactions

On the leaf surface of resistant and susceptible wheat genotypes, conidial germination in the three isolates ranged from 80% to 97% at 3 h after inoculation, with the highest percentage of germinated spores observed in 90-2. However, by 6 h, germination of conidia approached 100% in all isolate/genotype combinations. The average number of germ tubes formed per conidium on the leaf surface was not significantly different until 48 h after inoculation ($P<0.001$), at which time it was highest in 90-2 and lowest in Alg3-24 (Table 3-2). However, by 72 h, the average number of germ tubes per conidium was once again similar in all combinations of genotypes and isolates. No significant differences were observed in terms of the germ tube number when the same isolate was inoculated onto the different host genotypes (Table 3-2).

Similarly, the number of appressoria produced by each isolate was similar on both wheat genotypes, but differed between isolates at most time points (Table 3-3). By 3 h after inoculation, Alg3-24 had produced an average of 0.81 or 1.26 appressoria per conidium on 'Erik' and 6B662, respectively. In contrast, appressoria formed by 92-171R5 or 90-2 were rare or absent at this time. Throughout the remainder of the time-course, the number of appressoria produced was consistently higher in Alg3-24 than in 92-171R5 or 90-2, with the lowest values usually observed for 90-2 (Table 3-3). Therefore, while the mean number of appressoria produced by 90-2 on either host ranged from 0.47 to 0.80 at 24 to 72 h, in Alg3-24 these values ranged from 1.10 to 1.93. The differences between Alg3-24 and 90-2 were significantly different

Table 3-2. Mean number of germ tubes per conidium after inoculation of isolates Alg3-24, 92-171R5 and 90-2 of *Pyrenophora tritici-repentis* onto wheat line 6B662 and cultivar Erik.

		Time after inoculation (h)					
		3	6	12	24	48	72
Alg3-24	6B662[†]	2.56 a	2.14 a	3.03 a	2.50 a	2.23 b	2.67 a
	Erik	2.56 a	2.14 a	3.03 a	2.50 a	2.23 b	2.67 a
92-171R5	6B662	2.25 a	2.72 a	2.47 a	2.93 a	2.56ab	3.00 a
	Erik	2.00 a	2.90 a	3.13 a	3.00 a	2.90ab	2.73 a
90-2	6B662	2.11 a	2.93 a	2.90 a	3.50 a	3.33 a	3.03 a
	Erik	2.38 a	2.70 a	2.77 a	3.10 a	3.33 a	2.93 a

[†]Mean number of germ tubes was assessed in total 30 randomly selected conidia at different times after inoculation of 6B662 and 'Erik' with the various fungal isolates; ANOVA was ran with the effects of isolate*wheat genotype. Means followed by a common letter in the same column are not significantly different ($P < 0.001$) as determined by Tukey's Studentized Range (HSD) test.

Table 3-3. Mean number of appressoria per conidium after inoculation of isolates Alg3-24, 92-171R5 and 90-2 of *Pyrenophora tritici-repentis* onto wheat line 6B662 and cultivar Erik.

		Time after inoculation (h)					
		3	6	12	24	48	72
Alg3-24	6B662[†]	1.26 a	0.55ab	1.23 a	1.37ab	1.17ab	1.83 a
	Erik	0.81 a	0.93 a	1.23 a	1.93 a	1.47a	1.10ab
92-171R5	6B662	0 b	0.48ab	0.73 a	0.30 c	1.23ab	0.97ab
	Erik	0 b	0.43ab	0.90 a	0.87bc	1.07ab	1.03ab
90-2	6B662	0 b	0.07 b	0.53 a	0.47 c	0.50 b	0.80 b
	Erik	0.07 b	0.27ab	0.37 a	0.60bc	0.47 b	0.43 b

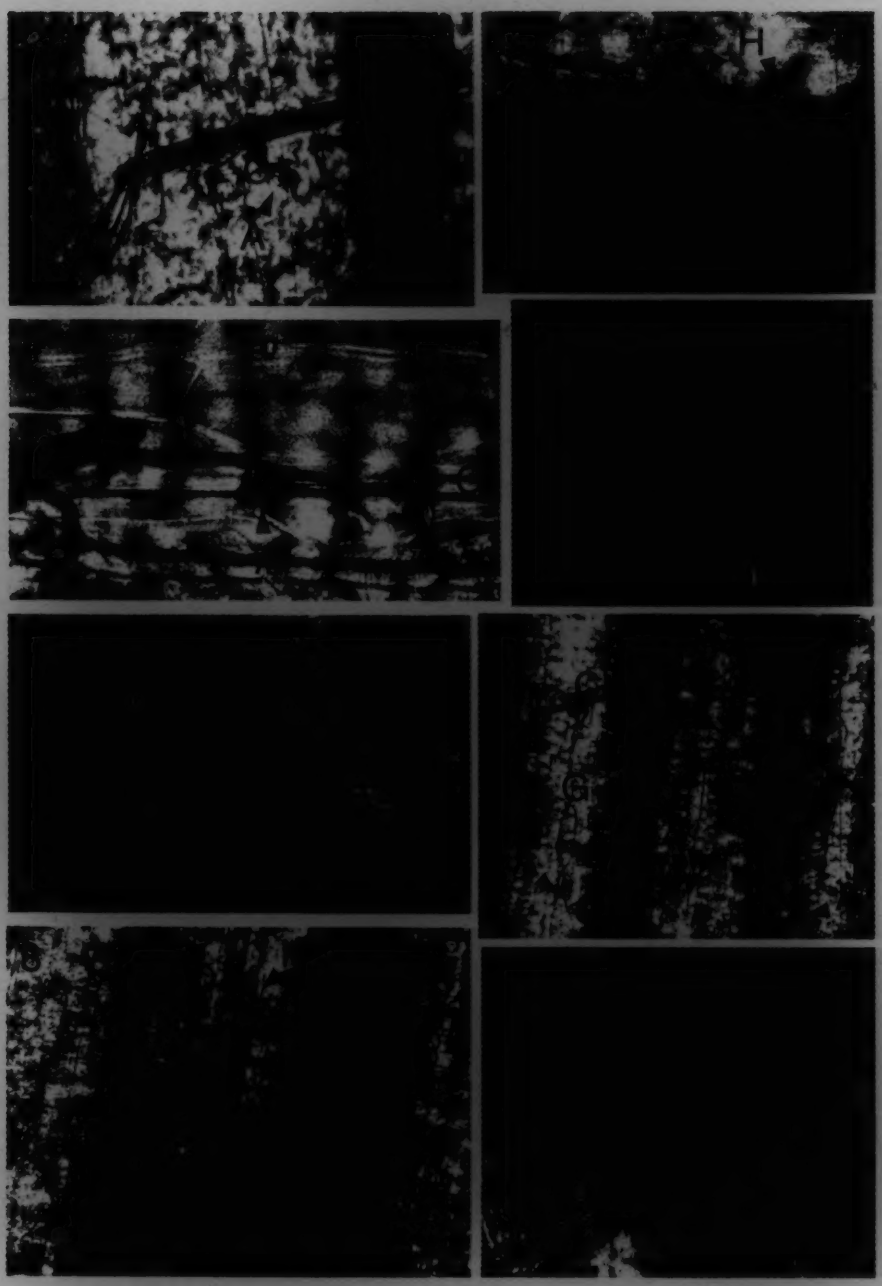
[†]Mean number of germ tubes was assessed in total 30 randomly selected conidia at different times after inoculation of 6B662 and 'Erik' with the various fungal isolates; ANOVA was ran with the effects of isolate*wheat genotype. Means followed by a common letter in the same column are not significantly different ($P < 0.001$) as determined by Tukey's Studentized Range (HSD) test.

($P<0.001$) at 0, 6 (for 6B662), 24, 48 and 72 h, with the values for 90-2 always lower (Table 3-3). The mean number of appressoria produced per conidium by 92-171R5 was usually intermediate, lying somewhere in between those observed for Alg3-24 and 90-2.

In Alg3-24, multiple appressoria were occasionally formed from a single germ tube, on both the Ptr ToxB-sensitive and insensitive hosts (Figs. 3-4 and 3-7). When a single appressorium was produced, it formed terminally on the germ tube (Figs. 3-5 and 3-6). The formation of multiple appressoria from a single germ tube was not observed in 92-171R5 (Figs. 3-11, 3-13 and 3-15) or 90-2 (Figs. 3-19 and 3-20), which developed only one appressorium per germ tube. Appressoria produced by all isolates were generally round or club-shaped. As previously reported for necrosis-inducing isolates of *P. tritici-repentis* (Larez et al. 1986; Loughman and Deverall 1986), appressoria of Alg3-24 usually developed on epidermal cell junctures (Figs. 3-3, 3-4, 3-5 and 3-6). Similarly, appressoria of 92-171R5 also formed mainly on epidermal cell junctures (Fig. 3-14), but were frequently detected on the stomatal complexes as well (Figs. 3-11, 3-15 and 3-16). In contrast, most appressoria produced by isolate 90-2 formed over the stomatal complexes (Figs. 3-21 and 3-22), although a few were also observed on epidermal cell junctures (Fig. 3-19). In several instances, it appeared as though germ tubes of 90-2 penetrated the stomata directly (Fig. 3-18).

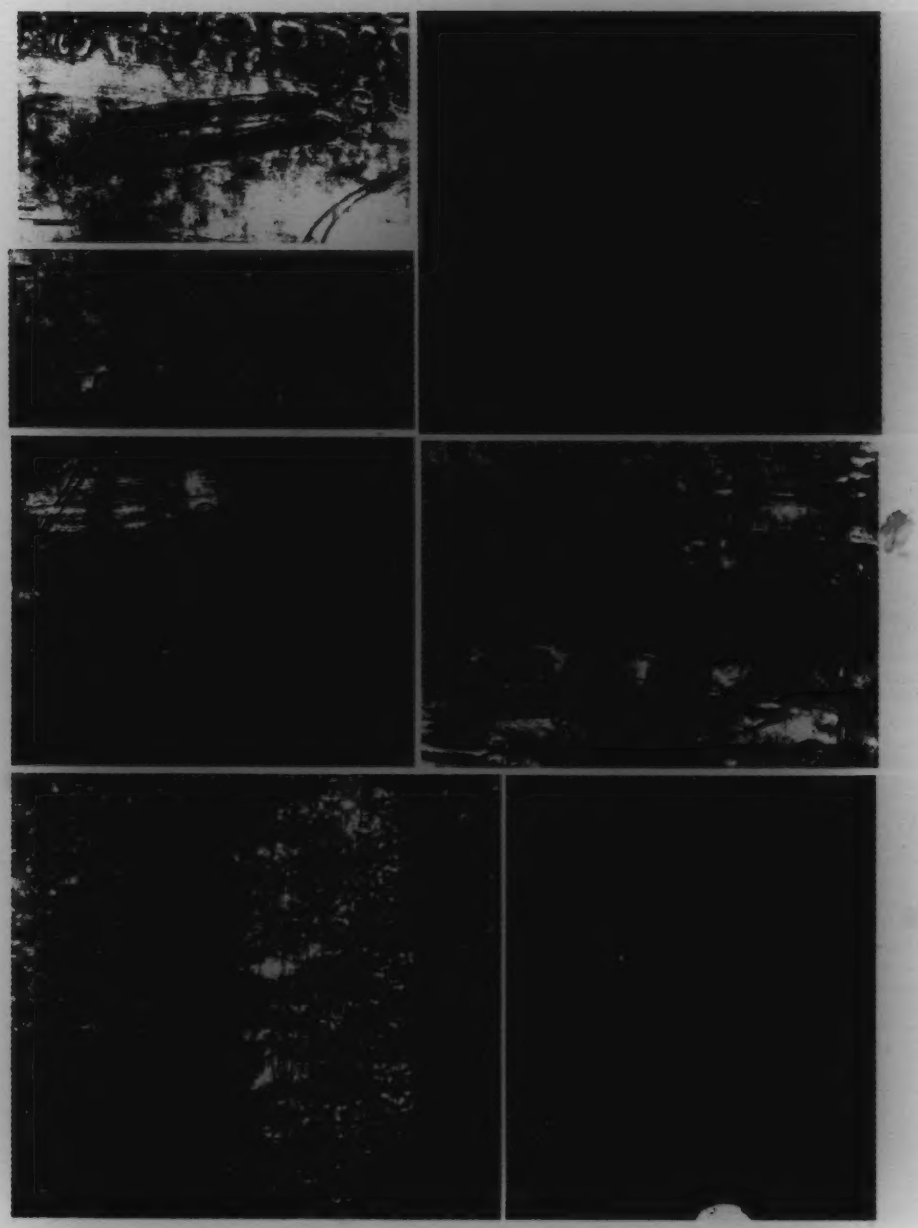
Following penetration by the fungal isolates, the epidermal cells stained deeply with cotton blue (Figs. 3-8, 3-9, 3-13, 3-14 and 3-15), suggesting that major physiological changes had occurred. Penetrations often resulted in the

Figures 3-2 – 3-9. Conidial germination and penetration of wheat epidermal cells by race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis*. (Fig. 3-2) Germinated conidium (C) on leaf surface of Ptr ToxB-sensitive wheat line 6B662, 3 h post-inoculation (p.i.). Note presence of appressoria (A) at end of germ tubes (G). Bar = 25 μm. (Fig. 3-3) Intracellular vesicle (V) and hypha (H) in epidermal cell of line 6B662 12 h p.i. Bar = 25 μm. (Fig. 3-4) Multiple appressoria (A) formed from one germ tube (G) on leaf surface of line 6B662, 24 h p.i. Bar = 25 μm. (Fig. 3-5) Papilla (P) formation by line 6B662 beneath appressorium (A), 48 p.i. Bar = 25 μm. (Fig. 3-6) Germinated conidium (C) on leaf surface of Ptr ToxB-insensitive wheat cultivar Erik, 6 h p.i. Note presence of appressoria (A) at end of germ tubes (G) and intracellular hypha (H). Bar = 25 μm. (Fig. 3-7) Multiple appressoria (A) formed from single germ tubes (G) on leaf surface of 'Erik,' 12 h p.i. Bar = 50 μm. (Fig. 3-8) Infected leaf epidermal cell (IE) 72 h p.i. of 'Erik.' Bar = 50 μm. (Fig. 3-9) Intracellular vesicle (V) and hypha (H) in infected epidermal cell (IE) of 'Erik' 48 h p.i. Bar = 25 μm. Tissue samples in Figs. 3-2 – 3-9 were stained with cotton blue and examined by light microscopy.



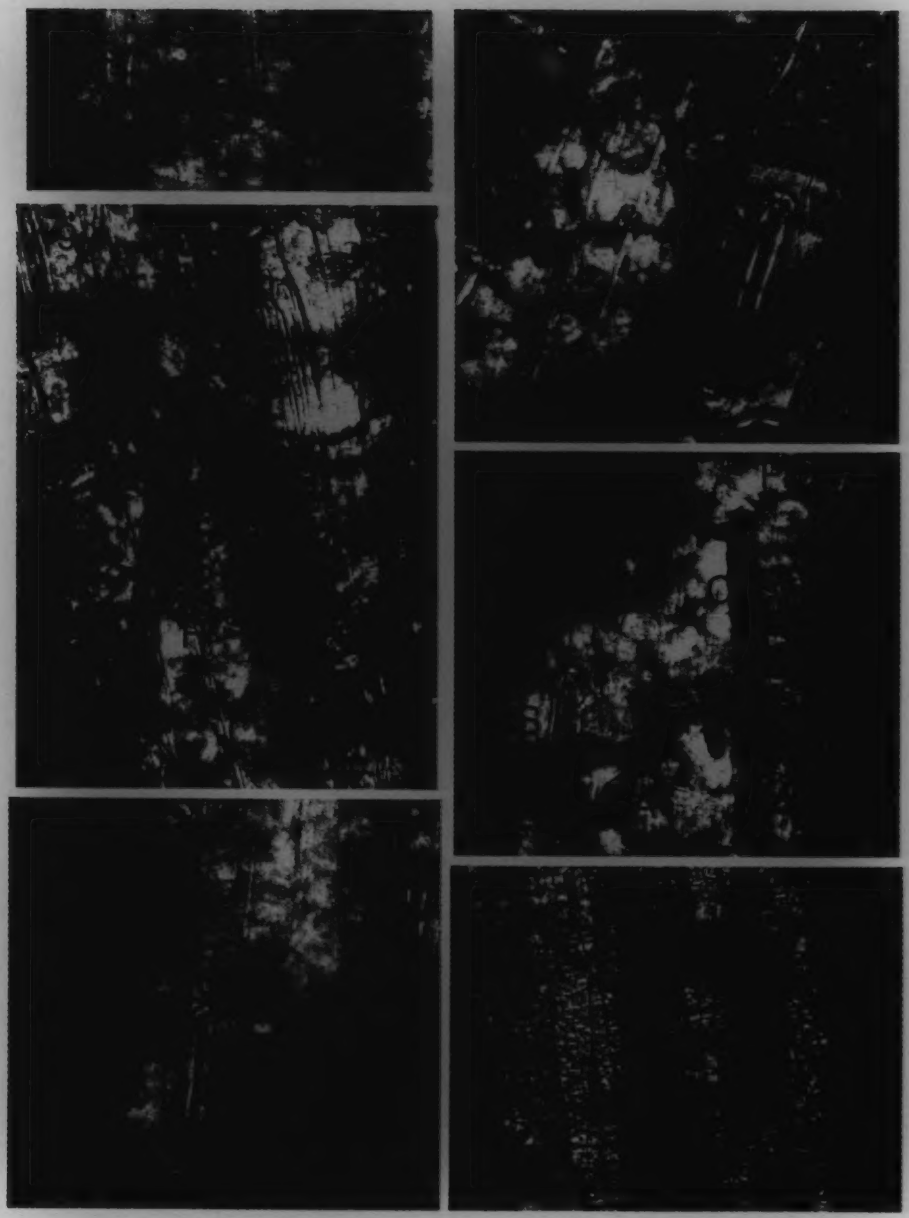
Figures 3-2 – 3-9

Figures 3-10 – 3-16. Conidial germination and penetration of wheat epidermal cells by low virulence race 5 isolate 92-171R5 of *Pyrenophora tritici-repentis*. (Fig. 3-10) Germinated conidia (C) and germ tubes (G) on leaf surface of Ptr ToxB-sensitive wheat line 6B662, 3 h post-inoculation (p.i.). Bar = 25 μm. (Fig. 3-11) Germinated conidium (C) on leaf surface of Ptr ToxB-insensitive wheat cultivar 'Erik', 12 h p.i. Note presence of appressorium (A) on stomatal complex (S). Bar = 25μm. (Fig. 3-12) Multiple germ tubes (G) emerging from one conidium (C) on leaf surface of 'Erik,' 6 p.i. Bar = 25 μm. (Fig. 3-13) Germinated conidium (C) on leaf surface of line 6B662, 12 p.i. Note presence of appressorium (A), intracellular hypha (H) and infected epidermal cells (IE). Bar = 25 μm. (Fig. 3-14). Papilla (P) formation by line 6B662 beneath appressorium (A), 24 h p.i. Bar = 25 μm. (Fig. 3-15) Germinated conidium (C) on leaf surface of 'Erik,' 24 p.i. Note presence of germ tubes (G) and appressorium (A) on stomatal complex (S). Bar = 25 μm. (Fig. 3-16) Germinated conidium (C) on leaf surface of 6B662, 24 p.i. Note presence of germ tube (G) and appressorium (A) on stomatal complex (S) for penetration. Tissue samples in Figs. 3-10 – 3-15 were stained with cotton blue and examined by light microscopy. The tissue sample in Fig. 3-16 was stained with Fluorescent Brightener 28 and examined by confocal microscopy.



Figures 3-10 – 3-16

Figures 3-17 – 3-22. Conidial germination and penetration of wheat epidermal cells by avirulent race 4 isolate 90-2 of *Pyrenophora tritici-repentis*. (Fig. 3-17) Germinated conidium (C) and germ tube (G) on leaf surface of Ptr ToxB-sensitive wheat line 6B662, 3 h post-inoculation (p.i.). Bar = 25 μm. (Fig. 3-18) Germinated condium (C) on line 6B662, 24 h p.i. Note penetration of stoma (S) by germ tube (G), Bar = 50 μm. (Fig. 3-19) Germinated conidium (C) on line 6B662 with germ tube (G) and formation of appressorium on epidermal cell juncture, 24 h p.i. An infected epidermal cell (IE) is also visible. Bar = 50 μm. (Fig. 3-20) Germinated conidium (C) with multiple germ tubes (G) on leaf surface of Ptr ToxB-insensitive cultivar 'Erik', 24 h p.i. Note presence of appressoria (A) at end of germ tubes, formed on epidermal cell junctures. A papilla (P) has developed beneath one appressorium. Bar = 50 μm. (Fig. 3-21) Intracellular vesicle (V) and hypha (H) in epidermal cell of 'Erik,' 24 h p.i. The vesicle is beneath an appressorium (A) formed over a stomatal complex (S). Bar = 50 μm. (Fig. 3-22) Appressorium (A) formation on stomatal complex on leaf of 'Erik,' 48 h p.i. Bar = 50 μm. Tissue samples in Figs. 3-17 – 3-22 were stained with cotton blue and examined by light microscopy.



Figures 3-17 – 3-22

formation of papillae beneath the appressoria (Figs. 3-5, 3-11, 3-14 and 3-20). The number of papillae formed by the Ptr ToxB-sensitive (6B662) and insensitive ('Erik') host genotypes was not significantly different after inoculation with Alg3-24, and fluctuated substantially at the various time-points (Table 3-4). Similarly, inoculation with 90-2 did not induce differential responses with respect to papilla formation in the hosts. At most time-points, 'Erik' produced greater numbers of papilla than 6B662 in response to inoculation with 92-171R5, but the difference was not significant ($P<0.001$). As papillae were counted based on the number induced per conidium, rather than per appressorium, direct comparisons cannot be made in terms of papilla formation in response to the different isolates, since the isolates produced significantly different numbers of appressoria (Table 3-3). Nevertheless, the development of papillae appeared greatest after inoculation with Alg3-24 and least in response to 90-2 (Table 3-4).

If the formation of host papillae was not induced, intracellular vesicles were often produced by the fungus. The intracellular vesicles were formed beneath the appressoria. The development of intracellular vesicles was most commonly observed in Alg3-24, which was followed by extension of the hyphae in the epidermal cells(Figs. 3-3 and 3-9). Intracellular vesicles formed by Alg3-24 were detected 6 h after inoculation (Fig. 3-3). Development of intracellular vesicles and hyphae was also observed in 92-171R5 (Fig. 3-13) and 90-2 (Fig. 3-21), although at a lower frequency. The formation of intracellular vesicles was detected 12 h after inoculation with 92-171R5, and 24 h after inoculation with 90-2. Disrupted mesophyll cells

Table 3-4. Mean number of papillae per conidium after inoculation of isolates Alg3-24, 92-171R5 and 90-2 of *Pyrenophora tritici-repentis* onto wheat line 6B662 and cultivar Erik.

		Time after inoculation (h)					
		3	6	12	24	48	72
Alg3-24	6B662[†]	0.27 a	0.17 a	0.43 a	0.37ab	0.47 a	0.63 a
	Erik	0.20 a	0.17 a	0.10ab	0.80 a	0.40ab	0.33ab
92-171R5	6B662	0 a	0 a	0.10ab	0.07 b	0.10ab	0.03 b
	Erik	0 a	0.07 a	0.20ab	0.40ab	0.17ab	0 b
90-2	6B662	0 a	0 a	0 b	0.07 b	0 b	0 b
	Erik	0 a	0 a	0 b	0.07 b	0 b	0 b

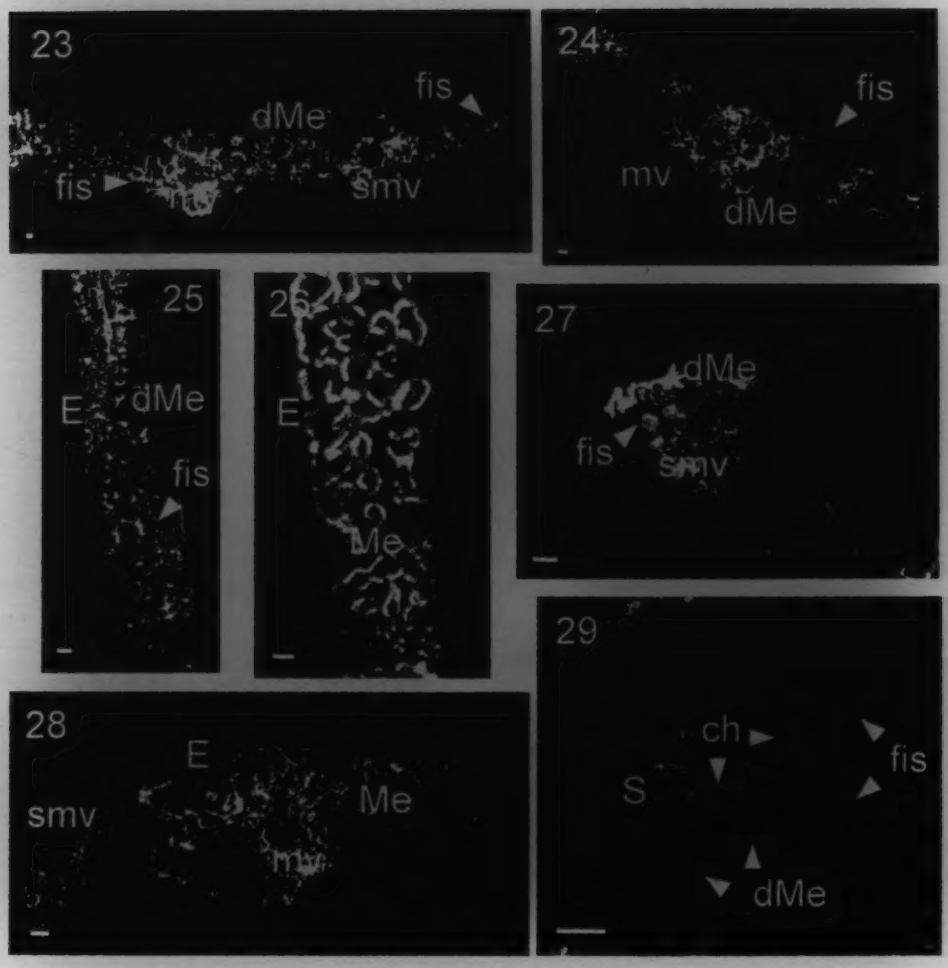
[†]Mean number of germ tubes was assessed in total 30 randomly selected conidia at different times after inoculation of 6B662 and 'Erik' with the various fungal isolates; ANOVA was ran with the effects of isolate*wheat genotype. Means followed by a common letter in the same column are not significantly different ($P < 0.001$) as determined by Tukey's Studentized Range (HSD) test.

[dMe] acquired an orange-red color when leaf sections were stained with Fluorescent Brightener 28. Disrupted regions of mesophyll were detected after inoculation with all three isolates, but the largest were observed in response to Alg3-24 (Figs. 3-23 and 3-24). Disrupted cells occurred both in the small necrotic centers of the lesions, as well as in the chlorotic zones surrounding them. The regions of disrupted mesophyll that developed after inoculation with 92-171R5 were similar to those induced by Alg3-24, but less extensive (Fig. 3-25). The smallest areas of disrupted mesophyll were observed in response to inoculation with 90-2. With this isolate, the inoculated tissue often resembled non-inoculated controls (Figs. 3-26 and 3-28), although localized zones of disrupted mesophyll were sometimes visible (Fig. 3-27). In response to inoculation with any of the isolates, mesophyll cells surrounding the infection site had a thickened appearance, and the intercellular spaces were filled with material (filled intercellular spaces [FIS], Figs. 3-24, 3-27 and 3-28). This was previously observed with a necrosis-inducing isolate (Dushnicky et al. 1998a; 1998b). The chloroplasts in the affected mesophyll appeared relatively intact, at least until 120 h after inoculation (Fig. 3-29).

3.4 Discussion

To our knowledge, this is the first microscopic examination of the interaction between Ptr ToxB-producing isolates of *P. tritici-repentis* and their wheat host. Furthermore, it is the first description of the behavior of a truly avirulent isolate (i.e. producing no known active forms of toxin) on toxin-sensitive and insensitive wheat

Figures 3-23 – 3-29. Transverse and longitudinal wheat leaf sections after inoculation with *Pyrenophora tritici-repentis*. (Fig. 3-23) Transverse section of a leaf of Ptr ToxB-sensitive wheat line 6B662 120 post-inoculation (p.i.) with isolate Alg3-24. Note stain retention of disrupted mesophyll cells (dMe). Bar = 20 μm. (Fig. 3-24) Transverse section of a leaf of Ptr ToxB-insensitive wheat cultivar 'Erik' 120 h p.i. with isolate Alg3-24. An area of disrupted mesophyll cells (dMe) and filled intercellular spaces (fis) is visible. Bar = 20 μm. (Fig. 3-25) Longitudinal section of 6B662 leaf 120 h p.i. with isolate 92-171R5, showing filled intercellular spaces (fis) and small area of disrupted mesophyll cells (dMe). Bar = 20μm. (Fig. 3-26) Longitudinal section of control 'Erik' leaf inoculated with water only. Bar = 20μm. (Fig.3-27) Transverse section of 6B662 leaf 120 h p.i. inoculation with isolate 90-2, showing very small area of disrupted mesophyll cells (dMe). Bar = 20μm. (Fig. 3-28) Transverse of 'Erik' leaf 120 h p.i. with isolate 90-2. Note small area of slightly affected mesophyll cells (Me) Bar = 20μm. (Fig. 3-29) Transverse section of 6B662 leaf 120 h p.i. with isolate 90-2, showing disrupted mesophyll cells (dMe), filled intercellular spaces (fis), and relatively intact chloroplasts (ch). Bar = 20 μm Tissue samples in Figs. 3-23 – 3-29 were stained with Fluorescent Brightener 28 and examined by fluorescence microscopy. Small mid-vein = smv, mid-vein = mv, epidermal cells = E.



Figures 3-23 – 3-29

genotypes. Generally, the infection process involving these isolates resembled that previously reported for necrosis-inducing (Ptr ToxA-producing) races of the fungus (Larez et al. 1986; Loughman and Deverall 1986; Dushnicky et al. 1996). The conidia germinated to produce germ tubes on which appressoria developed, and penetration of the epidermal cells usually resulted in the development of an intracellular vesicle by the fungus, or a papilla by the wheat host. Formation of an intracellular vesicle was followed by the development of intracellular hyphae in the epidermal cells. Finally, the fungus began intercellular growth in the mesophyll. A general model of the infection process involving Ptr ToxB-producing isolates of *Pyrenophora tritici-repentis* is shown in figure 3-30.

The infection process was similar in the susceptible (Ptr ToxB-sensitive) and resistant (Ptr ToxB-insensitive) wheat genotypes tested. Previous studies comparing compatible and incompatible interactions between wheat and necrosis-inducing isolates of *P. tritici-repentis* also found that the initial steps of infection were similar in both (Larez et al. 1986; Lamari and Bernier 1989a; Dushnicky et al. 1996). Comparable results have also been reported in other pathosystems, including those involving *Helminthosporium carbonum* and corn (Jennings and Ullstrap 1957), *Phytophthora infestans* and potato (Ferris 1955), and *Pyrenophora teres* and barley (Keeling and Banttari 1975). In the current study, the number of appressoria produced by individual isolates on the toxin-sensitive and insensitive host genotypes was not significantly different (Table 3-3). Likewise, each isolate induced a similar response in both host genotypes with respect to the formation of papillae (Table 3-4). It was

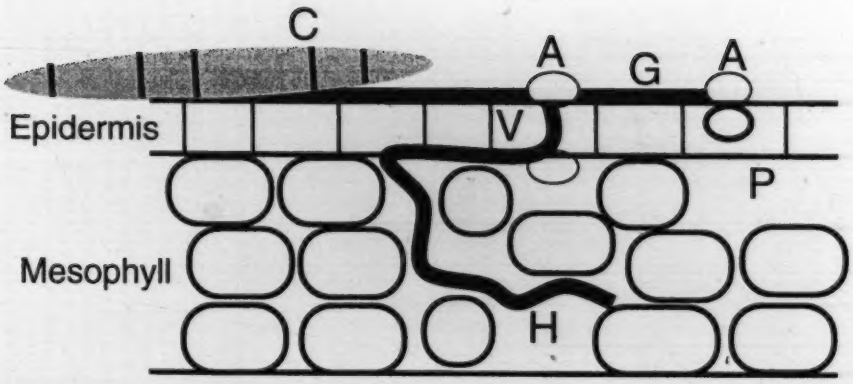


Figure 3-30. General model of the infection process involving Ptr ToxB-producing isolates of *Pyrenophora tritici-repentis*. Penetration and infection generally occur within the first 24 h after inoculation. A conidium (C) germinates, producing germ tubes (G) with one or more appressoria (A). A vesicle (V) may be formed upon penetration of epidermal cells, and occasionally the host will produce a papilla (P) in response to attempt penetration. If infection is successful, growth of hyphae (H) will continue intercellularly in the mesophyll cells.

only after isolates had penetrated the epidermis and had commenced growth in the mesophyll that differences in the responses of the two wheat genotypes became evident. The toxin-sensitive line 6B662 developed chlorosis in response to inoculation with virulent isolate Alg3-24 and low virulence isolate 92-171R5, while the insensitive cultivar Erik did not (Fig. 3-1). This differential response was also manifest as larger zones of disrupted mesophyll in 6B662, particularly after inoculation with Alg3-24, than in 'Erik' (Figs. 3-23 and 3-24).

The observation that *P. tritici-repentis* can penetrate the epidermal cells of incompatible wheat genotypes, grow intracellularly in these cells, and expand into the mesophyll has led to the suggestion that the virulence conferred by the Ptr toxins is superimposed over a basic pathogenic ability of the fungus (Lamari et al. 1998). However, the present study seems to suggest that this underlying pathogenic ability of *P. tritici-repentis* is not necessarily the same among isolates. For instance, while the number of appressoria formed did not differ when the same isolate was inoculated onto resistant and susceptible wheat genotypes, it did differ among isolates (Table 3-3). The most virulent isolate, Alg3-24, produced significantly higher numbers of appressoria per conidium than did the avirulent isolate, 90-2. Isolate 92-171R5 formed intermediate numbers of appressoria, which is consistent with its classification as low virulence (Strelkov et al. 2002). The speed of appressorium formation was also significantly different. While significant numbers of appressoria were formed by Alg3-24 at 3 h after inoculation, isolates 92-171R5 and 90-2 developed few if any appressoria until 6 h (Table 3-3). In contrast, features not

exclusively associated with parasitic ability, such as conidial germination rate and germ tube length, were similar in all isolates (Table 3-1).

In addition to the rate of formation appressoria, the location where they generally developed on the leaf surface also differed among isolates. Previous reports have indicated that most appressoria of necrosis-inducing isolates of *P. tritici-repentis* develop on epidermal cell junctures, with a smaller number forming on stomatal complexes (Larez et al. 1986; Loughman and Deverall 1986; Dushnicky et al. 1996). For example, Loughman and Deverall (1986) reported that only 1% of appressoria formed over the stomatal complexes, while Dushnicky et al. (1996) observed this in about 35-40% of the cases. In the present study, most appressoria formed by Alg3-24 and 92-171R5 developed on epidermal cell junctures (Figs. 3-6 and 3-14), while most appressoria formed by 90-2 were found on the stomatal complexes (Figs. 3-21 and 3-22). Dushnicky et al. (1996) suggested that the site for appressorium formation may vary as a result of environmental conditions, the fungal isolate used, or the method of inoculation. Given that in the current experiment the environmental conditions and method of inoculation were the same, the difference is likely attributable to the isolate. It is difficult to interpret the formation of appressoria over the stomatal complexes as a sign of reduced pathogenic fitness in 90-2, since the obligately parasitic rusts also form appressoria over the stomates. Nevertheless, in several instances, it seemed that germ tubes of 90-2 penetrated the stomata directly (Fig. 3-18), suggesting the possibility of direct penetration via natural openings in the host.

Regardless of what other features individual isolates of *P. tritici-repentis* may possess that contribute to their pathogenic ability, the capacity to produce one or more of the Ptr toxins is likely the most important (Strelkov and Lamari 2003). Indeed, acquisition of Ptr ToxA-producing ability by an avirulent isolate of the fungus was a sufficient condition for virulence (Ciuffetti et al. 1997). Similarly, the isolates used in the current study differed in their ability to transcribe the *ToxB* gene (Chapter 2), in addition to their differences with respect to the speed and quantity of appressorium formation; the transcription of *ToxB*, both in culture and in planta, was shown to be correlated with the virulence of the isolates, and was highest in Alg3-24 and lowest in 90-2. Nevertheless, factors such as the ability to produce appressoria may also contribute to the degree of pathogenicity exhibited by isolates of *P. tritici-repentis*.

Resistance to *P. tritici-repentis* in wheat appears to be a passive process, since the absence of receptors or targets for the toxins in the host is sufficient to confer resistance (Strelkov and Lamari 2003). Nevertheless, active resistance mechanisms have been implicated in the interaction. For instance, Loughman and Deverall (1986) noted that the formation of papillae was associated with the failure of *P. tritici-repentis* to penetrate wheat epidermal cells. Larez et al. (1986) found that the formation of papillae was also related to the restriction of hyphae in epidermal and/or mesophyll cells of oats. Nevertheless, while the formation of papillae may be sufficient to stop infection of individual host cells, it is, by itself, not sufficient to stop infection of the host as a whole. For instance, in the current study, line 6B662 and the

cultivar Erik produced papillae in comparable numbers in response to inoculation with Alg3-24, yet 6B662 was susceptible and 'Erik' was resistant (Table 3-4).

In addition to the development of papillae, lignification has also been implicated as a resistance mechanism in wheat against *P. tritici-repentis* (Dushnicky et al. 1998b). Using the same toxin-insensitive cultivar as in the current study ('Erik'), Dushnicky et al. (1998b) observed lignification and occlusion of the intercellular spaces of the mesophyll in response to inoculation with a necrosis-inducing isolate of the fungus. In contrast, no lignification was observed in a toxin-sensitive (susceptible) host genotype (Dushnicky et al. 1998b). In the present study, mesophyll cells surrounding the infection site had a thickened appearance, and the intercellular spaces were filled with material. However, this was observed both in the compatible and incompatible interactions (Figs. 3-23, 3-24, 3-25, and 3-29), and staining with toluidine blue O and acid fuchsin did not reveal any lignin-like material (results not shown). This apparent discrepancy may be related to differences in the experimental protocol employed, or perhaps more likely, to the fact that we inoculated plants with chlorosis-inducing isolates, resulting in the development of chlorosis rather than necrosis.

Virulence in *P. tritici-repentis*, although strongly linked to the ability to produce the Ptr toxins, appears to involve the interaction of additional factors, which impact the relative pathogenic ability of fungal isolates. This observation could explain the apparently contradicting results that have sometimes been reported with respect to inheritance of host resistance and toxin insensitivity in wheat (Lamari and Bernier 1989b; Gamba et al. 1998; Friesen et al. 2003; Friesen and Faris 2004).

Understanding of the additional factors contributing to pathogenicity may also help to explain the quantitative variation in virulence sometimes observed in isolates of *P. tritici-repentis* (Strelkov et al. 2002), at least in those associated with the production of Ptr ToxB. Although these factors are clearly secondary to the ability to produce the Ptr toxins, they may nevertheless provide important clues as to how basic compatibility between *P. tritici-repentis* and its wheat host is established.

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4.0 General Discussion

4.1 *ToxB* Gene Expression

In previous reports, the chlorosis-inducing, host-selective toxin Ptr ToxB was identified as a pathogenicity factor produced by the fungal pathogen, *Pyrenophora tritici-repentis* (Orolaza et al 1995; Strelkov et al. 1999). This toxin is encoded by the *ToxB* gene, homologs of which also occur in low virulence and avirulent isolates of the pathogen, which possess reduced or no toxin activity. In the current study, the *ToxB* homologs in these isolates were confirmed to be active genes (Chapter 2). However, gene expression differed in the isolates tested, and was generally correlated with virulence. Thus, *ToxB* expression was highest in virulent race 5 isolate Alg3-24, followed by low virulence race 5 isolate 92-171R5, and was lowest in avirulent race 4 isolate 90-2 (Chapter 2). This finding seems to reflect the importance of Ptr ToxB as a pathogenicity factor and its role in symptom development.

Unfortunately, antibodies were not available to assess production of the Ptr ToxB protein in vitro or in planta. Nevertheless, measurement of chlorophyll and carotenoid concentrations, which served as a measure of Ptr ToxB activity, confirmed strong chlorosis development in response to Alg3-24, weak chlorosis in response to 92-171R5, and little if any chlorosis in response to 90-2 (Chapter 2). Therefore, *ToxB* expression appears to be well-correlated to the presence and activity of the toxin protein, at least in the case of the two race 5 isolates (Alg3-24 and 92-171R5), in which the mature proteins encoded by the respective ORFs are identical (Strelkov and

Lamari 2003). In the case of 90-2, the situation may not be so simple. While the absence of chlorosis-inducing capacity in 90-2 could be related to the very low level of expression of the form of *ToxB* in this isolate (Chapter 2), it may have more to do with the activity of the Ptr ToxB protein that it encodes. The *ToxB* homolog from 90-2 shares only 86% similarity with the wild-type gene over the ORF (Strelkov and Lamari 2003). A recent study comparing the activity of heterologously expressed Ptr ToxB from isolates 90-2 and Alg3-24 indicated that the toxin from the former induces only very low levels of chlorosis in toxin-sensitive genotypes (Kim and Strelkov 2006).

Differences in *ToxB* gene expression among isolates of *P. tritici-repentis* are presumably related to differences in the transcriptional promoters of the various forms of the gene. Significant sequence differences were observed upstream of the ORF when the *ToxB* gene from another race 4 isolate, SD20, was compared with wild-type race 5 isolates from the United States (Martinez et al. 2004) and Algeria (Strelkov et al. 2006). Similarly, RT-PCR analysis using a number of different primers also indicated that substantial sequence differences also exist upstream of the *ToxB* ORF in 92-171R5, relative to the wild-type gene (Strelkov et al. 2006). However, the observed variation in *ToxB* expression between the isolates may also be related to differences in gene copy numbers. In virulent race 5 isolate Alg3-24, 8-10 copies of the gene are found. In contrast, there are only two copies of the *ToxB* homolog in low virulence race 5 isolate 92-171R5, and a single copy of the form of the gene in avirulent race 4 isolate 90-2 (Strelkov et al. 2006).

4.2 Pre-Penetration and Infection Processes

Microscopic examination of the infection process provided the first information on the histopathology and cytology of the interaction between Ptr ToxB-producing isolates of *P. tritici-repentis* and toxin-sensitive and insensitive wheat hosts. Rates of conidial germination were similar in all three isolates tested, and approached 100% within 6 h after inoculation onto the host leaves (Chapter 3), regardless of whether the isolates were virulent, low virulence or avirulent. Therefore, the viability of the conidia did not contribute to the differential pathogenicity of these isolates. Similarly, all three isolates penetrated and produced hyphae within the host epidermal cells (Chapter 3). These results are similar to the infection process described for necrosis-inducing (Ptr ToxA-producing) isolates of *P. tritici-repentis* (Larez et al. 1986; Lamari and Bernier 1989), and support the suggestion that the virulence bestowed by the Ptr toxins is superimposed over a basic pathogenic ability of the fungus (Lamari et al.1998).

Nevertheless, appressorium formation by the isolates appeared to be correlated to their level of virulence, with the highest average number of appressoria formed per conidium observed in Alg3-24, followed by 92-17R5, and the lowest number detected in 90-2. Furthermore, the speed of appressorium formation and infection was quickest in Alg3-24 (Chapter 3). These findings would seem to suggest that while all isolates of *P. tritici-repentis* may indeed share a basic pathogenic ability, the strength of this ability is not necessarily the same (S.E. Strelkov, personal communication). Other factors, in addition to Ptr ToxB, may contribute to their effectiveness as parasites.

Nevertheless, the fact that the avirulent isolate could be re-isolated from infected tissue one week after inoculation (Chapter 2), from essentially symptomless plants, confirms that Ptr ToxB plays a key role in disease development, regardless of other fungal qualities that may be at play.

4.3 Future Studies

Tan spot remains an important disease of wheat in parts of Canada and other regions of the world. In recent surveys of foliar wheat diseases in Manitoba, *P. tritici-repentis* was found to be one of the predominant pathogens of the spring (Gilbert et al. 2006) and winter wheat (Tekauz et al. 2006) crops, accounting for 32 and 63%, respectively, of all fungi isolated. Fortunately, in central Alberta, the disease was not identified in three wheat fields surveyed in 2005, although the closely related pathogen *Pyrenophora teres* was commonly found on barley (Orr et al. 2006). Tan spot often occurs as part of a leaf spotting complex in wheat, in conjunction with Stagonospora nodorum blotch (*Stagonospora nodorum*), Septoria tritici blotch (*Septoria tritici*), and spot blotch (*Bipolaris sorokiniana*) (Gilbert et al. 1998). Perhaps because of the close association between the disease causing organisms, it appears that *ToxA*, the gene encoding Ptr ToxA in *P. tritici-repentis*, was acquired from *Stagnospora nodorum* (Friesen et al. 2006). Therefore, improved understanding of the mechanisms of pathogenicity in tan spot may provide information that will be useful in understanding other pathogens involved in the wheat leaf spotting complex,

as well as closely related pathogens of other cereals. As such, further research is recommended on this pathosystem.

Many aspects of this additional research could follow from the work presented in the current thesis, some of which are immediately obvious. While we characterized expression of the *ToxB* gene in different isolates of *P. tritici-repentis*, we were not able to conduct a parallel study on Ptr ToxB protein levels, primarily because of a lack of appropriate antibodies. However, polyclonal antibodies against Ptr ToxB have now been produced, facilitating this aspect of the research. The antibodies could be used to detect the toxin protein in Western blots or enzyme-linked immunosorbent assays (ELISA), which will be particularly important for demonstrating Ptr ToxB production in isolate 90-2. Such research will also permit quantification and characterization of Ptr ToxB production in the various isolates, both in vitro and in planta. In terms of the microscopic analysis of the interaction, the availability of antibodies could facilitate immunolocalization studies, to determine the site of Ptr ToxB action in sensitive wheat cells. Alternatively, green fluorescent protein (GFP)-tagged toxin could be used to examine the localization of Ptr ToxB in host cells. These represent only a few of the many studies that may be necessary to fully understand Ptr ToxB, its mode of action, and its exact role in pathogenicity. Additional work will provide further insights into this important pathosystem.

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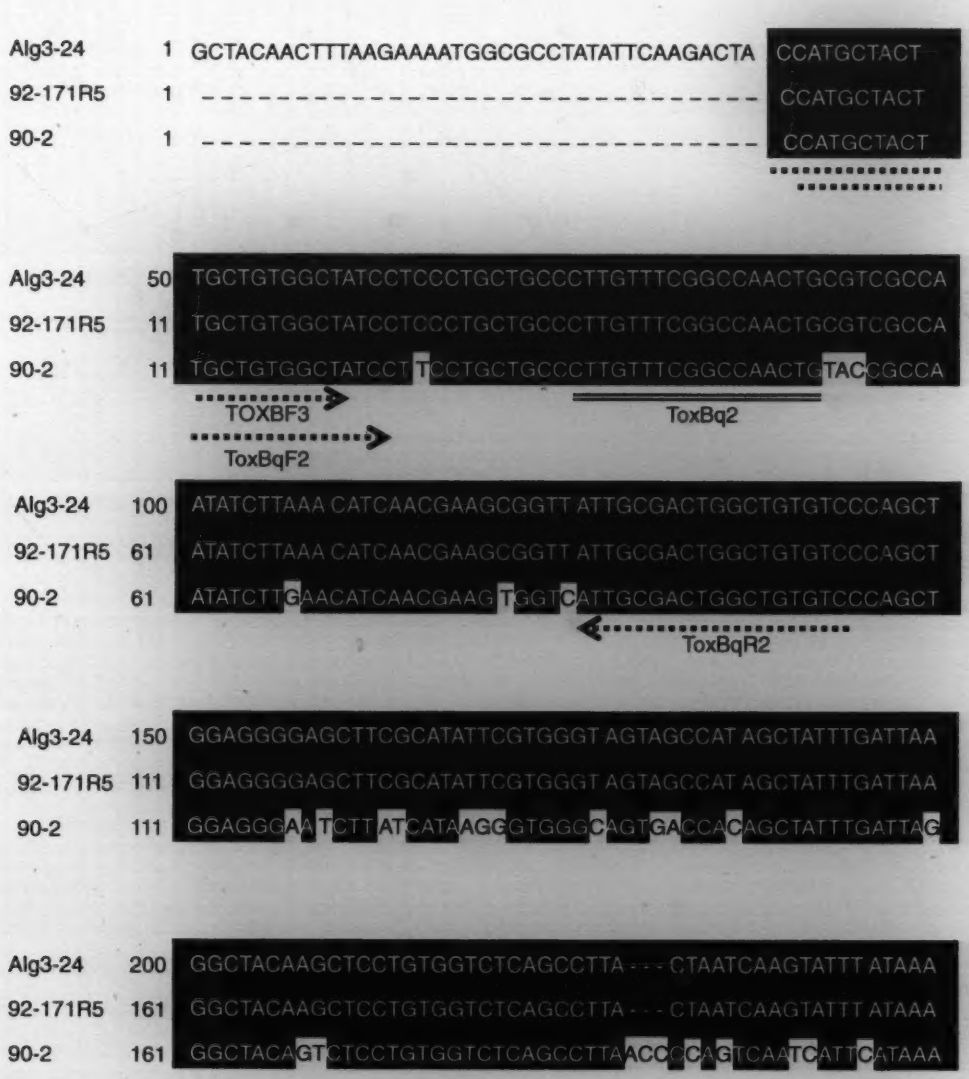
Appendix1: Nucleotide sequence comparison of *ToxB* cDNA from isolates Alg3-24, 92-171R5, and 90-2 of *Pyrenophora tritici-repentis*. The GenBank accession numbers for the sequences are: Alg3-24, AF483831; 92-171R5, AF483834; 90-2, AF484832. The locations of the TOXBF3/TOXBR3 primers used in RT-PCR, and the ToxBqF2/ToxBqR2 primers and ToxBq2 probe used in qRT-PCR, are indicated.



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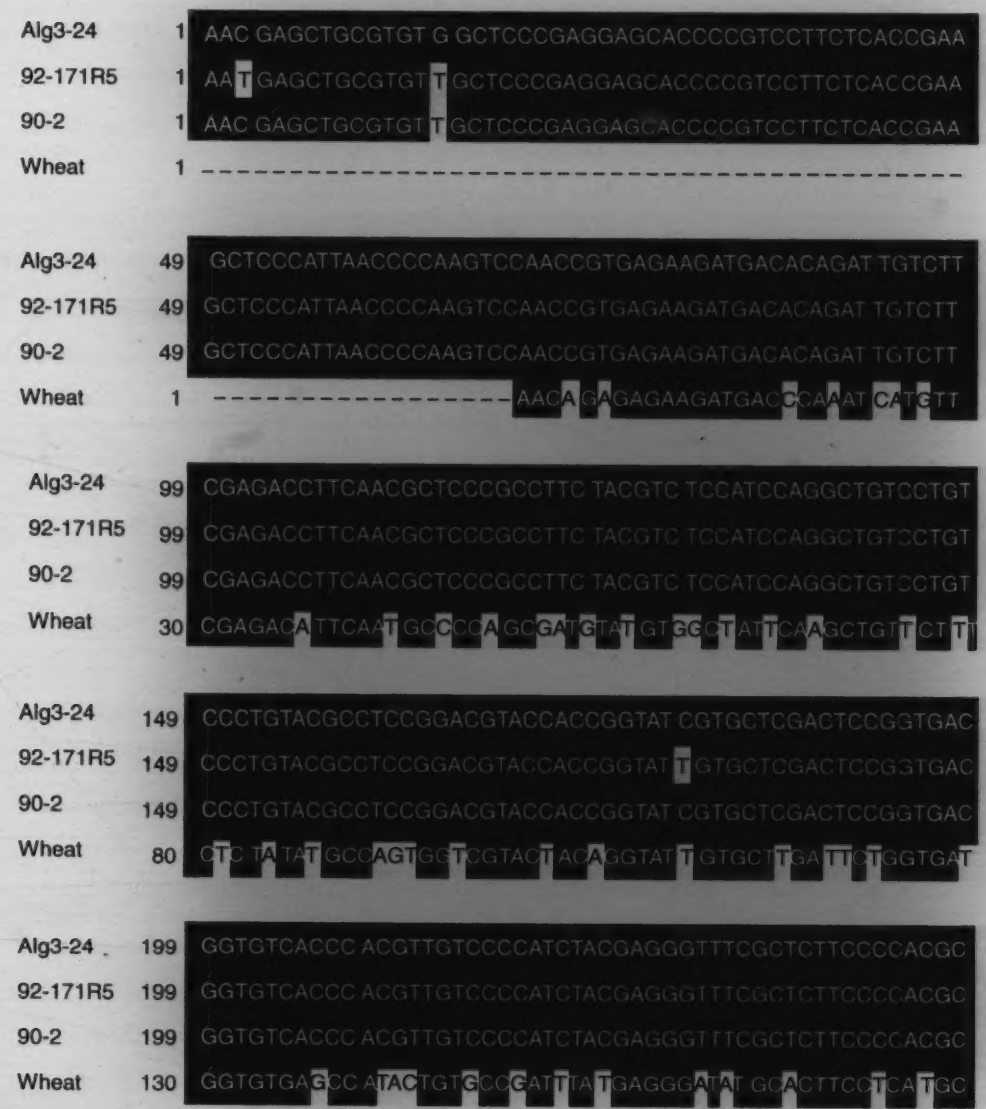
TOXBR3
Alg3-24 349 TTTATCTGGCATAGCACAGTTTTCCTC
92-171R5 ----------
90-2 ----------

Appendix2: Nucleotide sequence comparison of actin genomic DNA from isolates Alg3-24, 92-171R5, and 90-2 of *Pyrenophora tritici-repentis*. The GenBank accession numbers for the sequences are: Alg3-24, EF180087; 92-171R5, EF180088; 90-2, EF180089. The sequence from wheat actin (AY423548) is included for comparison. The solid line indicates the region coding for an intron. The locations of the ACTqF1/ACTqR1 primers and ACTq1 probe used in qRT-PCR are indicated.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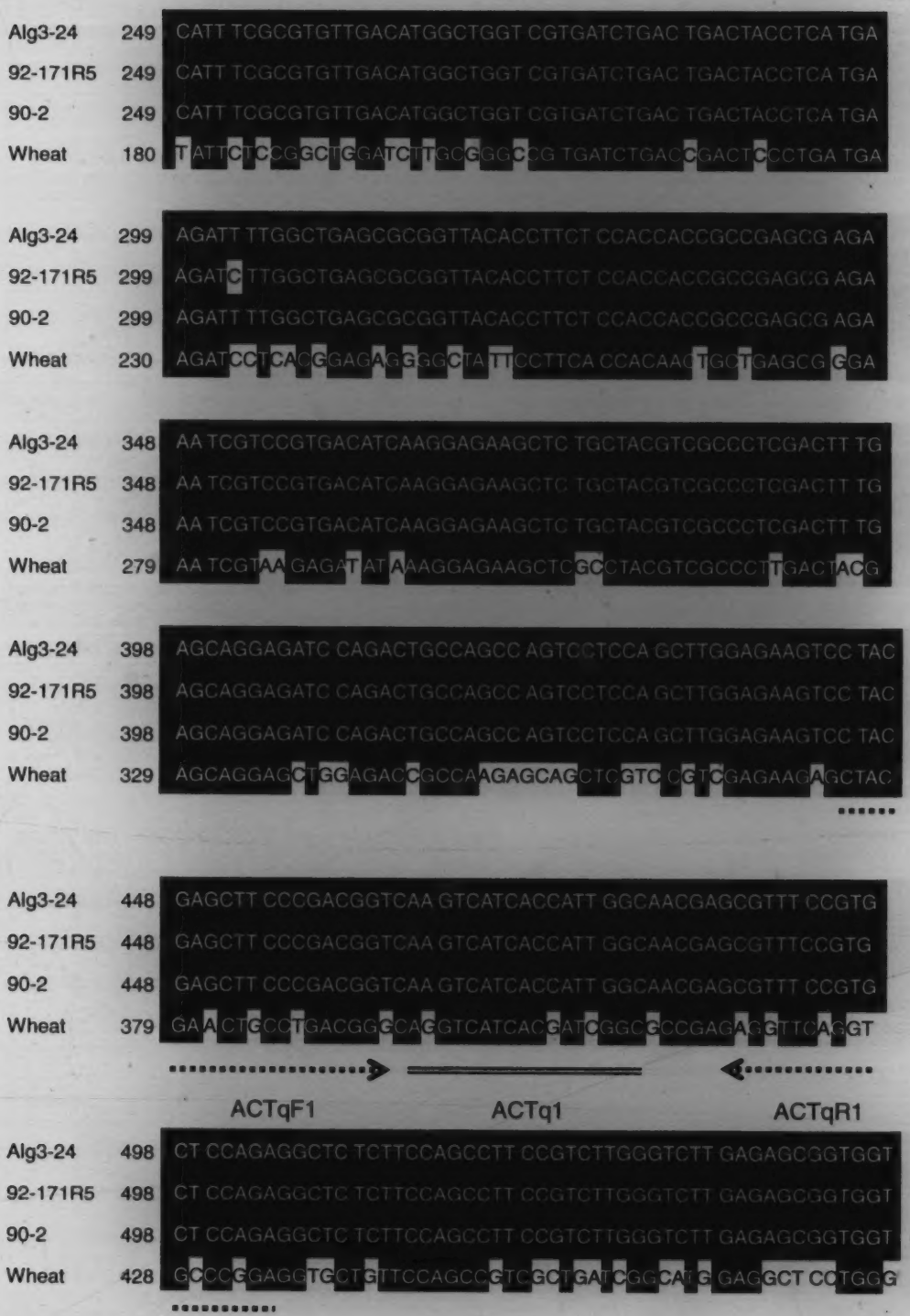
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ACTq1
ACTqR1
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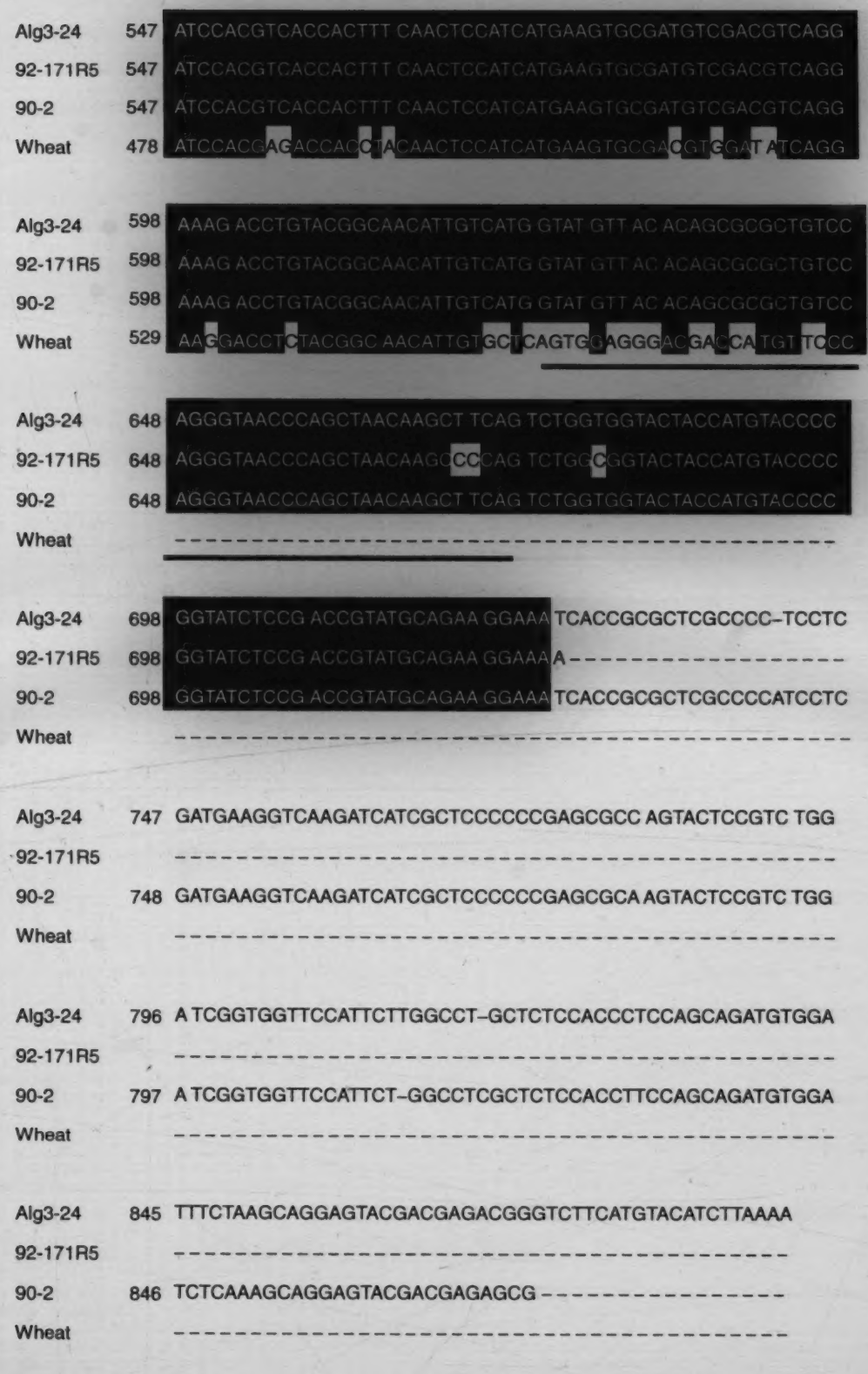
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